Pre-erythrocytic T cell immunity in malaria exposed Africans

Thesis

UNRESTRICTED

PRE-ERYTHROCYTIC T CELL IMMUNITY IN MALARIA EXPOSED AFRICANS

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ABSTRACT

Many believe that vaccines are the intervention with the greatest chance of reducing the worldwide burden of malaria. Irradiated sporozoites protect against subsequent malaria challenge, and a vaccine that mimics this stage might be effective. CD4 and CD8 T cells are thought to be crucial in protection, but studies of T cell immunity in malaria exposed donors are hampered by widespread nonresponsiveness, to malaria antigens. Most studies of natural T cell immunity measured lymphoproliferation alone, but I found that if 3 T cell assays were employed simultaneously (proliferation, overnight IFN-γ ELISPOT, and IFN-γ ELISPOT after 14 days culture), that T cell reactivity to circumsporozoite protein (CS) was considerably higher than when using proliferation alone. Responses to individual epitopes failed to correlate over the 3 assays, suggesting that they detect different memory T cell subsets. I investigated the nature of responder cells in the 3 assays, and found *ex-vivo* ELISPOT responses were mediated by $CCR7^-$ and cultured ELISPOT by $CCR7^+$ cells. I also found a $CD4^+CD38^+$ T cell subset that suppressed lymphoproliferation, and might contribute to malaria immunosuppression.

The immunodominant T cell epitope regions of CS are highly polymorphic, and I found that altered peptide ligand antagonism operated for variants of a CD4 T cell site. Less polymorphic antigens might be better vaccine candidates, one promising candidate being thrombospondin related adhesive protein (TRAP). I mapped novel CD4 T cell epitopes in TRAP, 16 of which were conserved. I found differences in TRAP T cell reactivity between East and West Africans, and adults and children, which is important since these

populations will be recipients of future malaria vaccines. I investigated whether IFN-γ ELISPOT responses to TRAP correlated with protection, since IFN-γ is thought to be the mechanism by which T cells exert their protective effect, but no protection was found.

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ABBREVIATIONS

aa	amino acid residues
Ab	antibody
Ag	antigen
ALP	alkaline phosphatase
APC	antigen presenting cell
APL	altered peptide ligand
BSA	bovine serum albumin
CD	cluster designation (e.g., CD45RB, CD38)
CPM	counts per minute
CS	circumsporozoite protein
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EE	exoerythrocytic
EIR	entomologic innoculation rate
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
EMP1	erythrocyte membrane protein 1
FACS	fluorescence activated cell sorter
FBS	foetal bovine serum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAGs	glycosaminoglycans
G6PD	glucose 6 phosphate dehydrogenase
GRP	glucose regulated protein
[^{3}H]	tritiated thymidine
HCl	Hydrogen chloride
HIV	human immunodeficiency virus
HLA	human histocompatibility leukocyte antigen
HPLC	high pressure liquid chromatography
hr	hour(s)
HSP	heat shock protein
IFN	interferon (e.g., IFN-γ)
IL	interleukin (e.g., IL-2)
IU	international unit
iNOS	inducible nitric oxide synthase
KO	knock out
LFA	lymphocyte function associated antigen
LSA	liver stage antigen
M	molar
mAb	monoclonal antibody
MEM	minimum essential medium
min	minutes
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
MLR	mixed lymphocyte reaction
MPC	magnetic particle concentrator
MSP	merozoite surface protein
MVA	modified vaccinia Ankara
NaCl	sodium chloride
ND	not determined

nm	nanometers
NK	natural killer
NO	nitric oxide
NS	not significant
OD	optical density
p	probability
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pb	*Plasmodium berghei*
Pf	*Plasmodium falciparum*
Pv	*Plasmodium vivax*
Py	*Plasmodium yoelii*
*Pf*EXP-1	*P. falciparum* exported protein-1
PHA	phytohaemagglutinin
PPD	purified protein derivative
PVM	parasitophorous vacuole membrane
RESA	ring-infected erythrocyte surface antigen
RFLP	restriction fragment length polymorphism
RT	room temperature
SALSA	sporozoite and liver stage antigen
SE	standard error
SFC	spot forming cells
SHEBA	sporozoite hepatocyte binding antigen
STARP	sporozoite threonine- and asparagine-rich protein
TCR	T cell receptor for antigen
Th1	T helper 1
Th2	T helper 2
TNF	tumour necrosis factor
TRAP	thrombospondin related adhesive protein
TT	tetanus toxin
u	unit
VLP	virus like particle
VS or vs	versus

CHAPTER 1

INTRODUCTION

1.1

MALARIA PERSPECTIVE

Two major breakthroughs in malaria research more than a century ago led to the award of Nobel Prizes. One to Alphonse Laveran for his discovery that malaria is caused by a protozoan parasite in the blood (Laveran, 1880), and another to Sir Ronald Ross who found that malaria is transmitted by mosquitoes (Ross, 1897). Despite such seminal discoveries over 100 years ago, we are still not winning the battle against this worldwide tyrant. One third of the world's population remains exposed to the risk of malaria, and hundreds of millions of clinical cases occur each year. The vast majority of disease and death occurs in sub-Saharan Africa (WHO, 1992), where it is estimated that malaria is responsible for up to 2 million deaths a year, mainly in children (WHO, 1995).

Resistance to antimalarial drugs has now been described to almost all those available (White, 1998), and continues to increase. Newer treatments are more expensive, and there is little impetus for drug companies to invest in developing drugs that will be affordable in developing countries. Anophelene mosquito resistance to commonly used insecticides precludes the use of large scale vector control programmes, once thought to be an effective strategy for malaria control. Permethrin treated bednets are highly effective in reducing childhood mortality, however the long term sustainability of such programmes is debatable (Snow *et al.*, 1997). These factors, compounded by inadequate infrastructure and resources for delivery and monitoring of control measures, highlight the difficulties involved in the fight to control this parasite.

Alternative solutions must be sought, and many believe that malaria vaccines may be the intervention with the greatest chance of reducing the worldwide burden of this disease. (Doolan and Hoffman, 1997, Kwiatkowski and Marsh, 1997, Miller and Hoffman, 1997).

Intense research efforts have been made in the last 15 years towards this ultimate goal. The studies in this thesis aimed to further our understanding of the development and nature of natural immunity in malaria exposed individuals, which in turn might improve the design of future malaria vaccines.

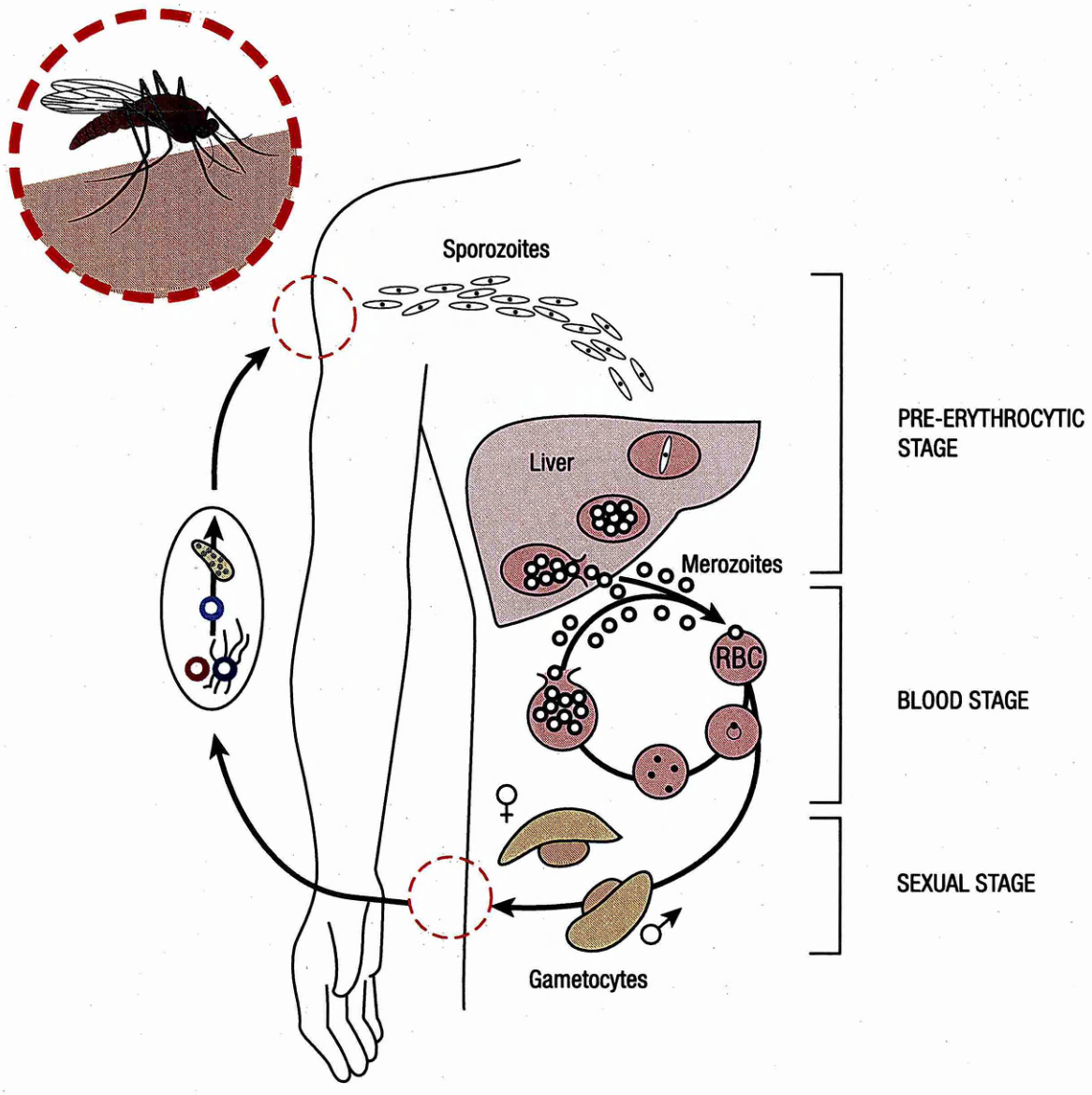
1.2

MALARIA LIFE CYCLE (Figure 1.1)

The infected female anophelene mosquito injects the infective sporozoites from her salivary glands into the host blood stream at the time of taking a blood meal. It is estimated that the mean number of sporozoites injected at any one time ranges between 10 and 100 (Rosenberg *et al.*, 1990, Ponnudurai *et al.*, 1991). These pass to the host liver within minutes, and 5-10% of the initial innoculum successfully penetrate hepatocytes and begin dividing (Ferreira *et al.*, 1986a). They form pre-erythrocytic schizonts in which up to 30,000 daughter cells called merozoites develop. After a minimum of $5^1/_2$ days for *P. falciparum* the merozoites lyse the hepatocytes and enter the red blood cells within the hepatic sinusoids (Nardin and Nussenzweig, 1993). The stage from sporozoite inoculation to release into the blood stream comprises the pre-erythrocytic stage of infection.

The blood stage commences with the entry of merozoites into host red blood cells. Here they divide to become schizonts containing 10-30 merozoites, which subsequently rupture and release merozoites into the blood, each of which is capable of entering a host red blood cell. This asexual stage leads to an exponential increase in parasites, with up to 10 fold increases in parasitaemia every 48 hours. It is the intermittent release of merozoites from infected red blood cells which gives rise to the symptoms, and is responsible for the cyclical pattern of disease. After a variable period of infection, a small proportion of merozoites enter the sexual stage by differentiating into gametocytes within red blood cells as male (micro-) gametes and female (macro-) gametes. If ingested by a feeding mosquito, they enter the mosquito midgut where they mature and fuse to form zygotes.

Figure 1.1



The life cycle of malaria indicating the 3 main stages for vaccine development: pre-erythrocytic stage, blood stage and sexual stage.

Zygotes develop into ookinetes which invade midgut epithelial cells and develop into thousands of sporozoites. These enter salivary glands from whence they transmit infection when the mosquito feeds on a human host (Sinden *et al.*, 1996).

1.3

MALARIA VACCINES

Malaria vaccines may be broadly divided into 3 main groups according to the parasite stage they are targeted at: pre-erythrocytic stage, blood stage and sexual stage. Different antigens are expressed at each of these stages, and an effective vaccine may need to combine antigens from each stage. Many conventional vaccines against infectious diseases use live attenuated or inactivated organisms. This strategy is not applicable to malaria vaccine design since it is not logistically possible to produce sufficient attenuated organisms. The rapid development of molecular technology and genome mapping in recent years has opened up the possibility of constructing malaria epitope and antigen based vaccines, and the race is now on to develop a recombinant vaccine that works.

1.3.1

Pre-erythrocytic Stage Vaccines

The rationale for development of pre-erythrocytic stage vaccines is based upon early observations in the murine model and humans that injection with radiation attenuated sporozoites can induce sterile protection against subsequent malaria challenge (Nussenzweig *et al.*, 1969, Clyde *et al.*, 1973, Clyde *et al.*, 1975, Rieckmann *et al.*, 1979). Irradiated sporozoites develop only partially inside hepatocytes (Sigler *et al.*, 1984), and do not mature fully or produce blood stage infection. The protection is not strain specific, and lasts at least nine months in humans (Hoffman *et al.*, 1996). Sporozoite production on a large scale is not feasible, but a vaccine that mimics the sporozoite effect is thought to be an achievable goal.

It is probable that irradiated sporozoites induce immunity against hundreds or thousands of antigens, which may explain why protection is heterologous in nature. Extensive efforts

have focused on identifying the antigenic targets at the pre-erythrocytic stage, and characterising the nature of the protective immune response. Numerous pre-erythrocytic antigens have now been identified, although most effort has focused on just one pre-erythrocytic antigen called the circumsporozoite (CS) protein. As new pre-erythrocytic antigens emerge, and new vaccine strategies are developed, the possibility of developing an effective vaccine against this stage increases.

A fully effective pre-erythrocytic vaccine, that prevents the development of the symptomatic blood stage, might be highly suitable for the non-immune traveller. However, such a vaccine might not be suitable for naturally exposed donors in areas of intense malaria transmission, where adults are semi-immune. A totally effective pre-erythrocytic vaccine would prevent the development of this natural immunity, and if pre-erythrocytic immunity waned with time, a vaccination programme could paradoxically lead to an increase in the incidence of severe disease and death. However, a partially effective pre-erythrocytic vaccine might reduce the number of parasites reaching the blood stage, thus reducing the parasite burden, and possibly reducing mortality as a result of decreased parasite loads, whilst allowing immunity to develop.

1.3.2

Blood Stage Vaccines

The blood stage is able to progress exponentially in the unprotected host, leading to the symptoms of fever and rigors, and later more serious complications such as anaemia, coma, renal failure and death. Many of the life threatening complications are thought to be due to sequestration of the parasite in tissue microvasculature where they cause obstruction and destruction due to the release of harmful cytokines and inflammatory mediators.

Vaccines designed against the blood stage aim to prime the individual against blood stage antigens in an attempt to contain the disease and prevent exponential parasite growth. The primed response can then be boosted by infection, thereby controlling disease progression before more serious complications develop. This would be rather like the state of natural

immunity which develops in those who are exposed throughout their lives, since they often carry blood stage parasites, but have no symptoms of disease. The goal of a blood stage vaccine would thus to be to prevent disease rather than infection. Unfortunately there has been reluctance to invest money into blood stage vaccine development for use in humans because they do not provide sterile protection, and are of little use to the "at risk" traveller. There is little commercial interest in a vaccine designed exclusively for use in the developing world, and hence little motivation to take such products to human trials.

Early experiments demonstrated that monkeys immunised with *P. knowlesi* merozoites in complete Freund's adjuvent can rapidly clear homologous and heterologous blood stage challenge (Mitchell *et al.*, 1975). Blood stage protection is mainly antibody mediated, but both IFN-γ production and T cell proliferation in response to blood stage antigens are also associated with protection (Miller *et al.*, 1998, Good *et al.*, 1998). Numerous antigens are expressed throughout the blood stage of infection and have differing roles in the life cycle. Obvious targets are those expressed on the merozoite surface, and those released by the merozoite during red blood cell invasion (attachment, orientation and penetration). The most intensively studied blood stage vaccine candidate is merozoite surface protein-1 (MSP-1) which is the major surface protein on the merozoite (Holder and Freeman, 1982, Holder and Blackman, 1994). Antibodies against a specific region of MSP-1 have been shown to correlate with protection against clinical episodes of malaria in naturally exposed donors (Egan *et al.*, 1996).

Parasitisation of red cells leads to a change in the antigens expressed on the red cell surface and the red cell structure (Crandall and Sherman, 1994). Infected red cells bind to the endothelium in a receptor-specific manner to prevent them from being killed on passing through the spleen. *P. falciparum* erythrocyte membrane protein 1 (*Pf* EMP1) is the molecule primarily involved in binding to vascular endothelium, and would seem to represent an ideal vaccine candidate antigen. Unfortunately the parasite has developed an extraordinary capacity for phenotypic variation of *Pf*EMP1 with up to 150 copies per parasite genome (Su *et al.*, 1995, Smith *et al.*, 1995). Reactivity in naturally exposed donors

to *Pf*EMP1 is variant specific, and while this huge variation provides an excellent immune escape mechanism for the parasite (Bull *et al.*, 1998), it makes *Pf*EMP1 a less than ideal vaccine candidate. Since the variant gene family that encodes *Pf*EMP1 has been cloned (Baruch *et al.*, 1995, Su *et al.*, 1995), the hope is to find relatively conserved regions to which Abs might be induced. The transitory nature of expression of many of these ligands make them less than ideal as vaccine candidates, and no protein has yet been identified that is continually expressed on the surface of merozoites and involved in signalling.

Patarroyo and colleagues developed a vaccine called SPf66 comprised of 3 putative blood stage antigen peptides and the NANP peptide from the circumsporozoite protein (Patarroyo *et al.*, 1987). Studies in Columbian adults and children (Valero *et al.*, 1993) and in Tanzanian children (Alonso *et al.*, 1994) suggested that the vaccine can prevent approximately one third of clinical episodes of malaria, but subsequent field trials in The Gambia, Thailand and Tanzania failed to substantiate these protective effects in children (d'Allesandro *et al.*, 1995, Nosten *et al.*, 1996, Acosta *et al.*, 1999).

1.3.3

Sexual Stage Vaccines

Antibodies are generated against gametocyte antigens during the course of natural infection (Graves *et al.*, 1990), and it is possible to immunise the mammalian host against the sexual stage (Carter and Chen, 1976, Gwadz, 1976). Sexual stage or transmission-blocking vaccines contain gamete, zygote and ookinete surface proteins and ookinete secreted proteins. They induce Abs that are ingested by the mosquito with the blood meal and prevent the development of the sexual stages, thus arresting the parasite life cycle within the mosquito. Infected humans produce Abs to a 230 kDa gametocyte antigen which has been correlated with suppression of infectivity in mosquito feeding experiments (Quakyi *et al.*, 1987), but whether this response reduces malaria transmission in the field is not known.

These vaccines will not prevent the disease process in the person being vaccinated, but will block the transmission of parasite from the infected host. As such they are described as altruistic vaccines, and are aimed at reducing malaria within the community with no benefit for the individual recipient. They could be used to prevent the spread of parasites that are drug resistant, or for epidemic control in areas of unstable transmission. They might also be useful in eradication programmes in small communities or islands where transmission rates are low, probably in combination with other strategies such as insecticide programmes. Some argue that sexual stage vaccines might also be of benefit in high transmission areas since the reduction in transmission intensity might have a similar impact on morbidity and mortality to that seen following the implementation of bednet control programmes (Alonso *et al.*, 1991, Snow *et al.*, 1999).

1.3.4

Malaria Vaccine Field Trials

The final stage in testing a candidate vaccine will require field trials in malaria endemic regions to look for protective efficacy. The design and implementation of such studies is fraught with problems. Most of the countries where malaria is a major health problem are very poor, with little health surveillance infrastructure for assessment of outcomes. Transmission blocking vaccine trials are severely limited by the fact that they need to be tested in isolated communities where the influx of new mosquitoes is absent. An ideal site would be an archipelago of small islands, although the results of such a trial may not be relevant to mainland Africa where the malaria burden is greatest.

The design of the trial is the next problem, and even the definition of end points has proved challenging. Pre-erythrocytic vaccines, which are intended to arrest development before the blood stage, are relatively straightforward to assess compared to blood stage vaccines which are designed to reduce the blood stage disease severity. If someone has symptomatic malaria then one is ethically obliged to treat them, and thus it is impossible to assess the disease prevention efficacy of a blood stage vaccine in the field. Defining a case of malaria has proved more difficult than it might at first seem since asymptomatic parasitaemia is

common in high transmission regions, and an episode of fever may be due infection other than malaria, even in parasitaemic individuals. The definition of a clinical episode must be tailored according to the local epidemiological circumstances (Smith *et al.*, 1994). There is the issue of how to assess one of the most important outcomes of vaccine implementation, namely the impact on mortality. This requires very large numbers of volunteers, and cannot be assessed very easily in a closely monitored population who will be treated each time they are found to have symptomatic disease.

The finding that permethrin-impregnated bednets reduce mortality despite an unchanged infection rate (Alonso *et al.*, 1991) highlights the complexity of assessing the impact of intervention measures. This is further complicated by the fact that different levels of transmission are associated with different patterns of disease (Marsh, 1992, Miller *et al.*, 1994, Snow *et al.*, 1997). Thus, in areas of intense transmission (>100 infectious bites / individual per yr), such as parts of Kenya, the major manifestation of severe malaria is severe anaemia seen mainly in the first few years of life. In areas of lower transmission (<15 infectious bites / individual per yr), such as The Gambia, cerebral malaria in 2 to 4 year old children is more common. There are concerns that a reduction in the transmission rates due to vaccination might even increase the mortality, since there is evidence that malaria mortality is lower in high transmission areas (Snow *et al.*, 1997).

Despite these difficulties, several vaccine candidates have been tested in field studies. The only malaria vaccine candidate to reach phase III trials to date is SPf66 (Valero *et al.*, 1993, Alonso *et al.*, 1994, d'Allesandro *et al.*, 1995, Nosten *et al.*, 1996), and Phase I, IIa and IIb trials of the candidate vaccine RTS,S are ongoing in The Gambia (Doherty *et al.*, 1999).

1.4

PRE-ERYTHROCYTIC ANTIGENS

The studies described in this thesis are of pre-erythrocytic T cell responses, and thus the other stages of malaria infection will not be discussed further. The fact that sporozoite immunisation is not strain specific, and is efficacious in all humans who receive an optimal

dose, suggests that the immune response is directed against multiple pre-erythrocytic antigens / epitopes. Hence considerable efforts have been made to identify new parasite proteins expressed at the pre-erythrocytic stage of infection, which might be incorporated into vaccine constructs to enhance the immunogenicity.

Precisely how pre-erythrocytic antigens are recognised by the immune system is not completely understood. Sporozoites are injected by the bite of a female *Anopheles* mosquito and quickly localise to the liver sinusoids. Here they must cross the endothelium into the space of Disse before entering hepatocytes. Some studies suggest that sporozoites are arrested by Kupffer cells in the liver, although the evidence for this is contradictory (Sinden and Smith, 1982, Meis *et al.*, 1983, Hollingdale, 1988, Vreden, 1994). A major role for Kupffer cells is unlikely since sporozoites interact poorly with Kupffer cells *in vitro* (Seguin *et al.*, 1989), and Kupffer cell depletion has little effect on sporozoite infectivity *in vivo* (Vreden, 1994). An interaction between sporozoites and macrophages has been suggested, and phagocytosis by macrophages may be involved in induction of protective immunity at this stage (Danforth *et al.*, 1980, Vanderberg *et al.*, 1990).

Sporozoites bind directly to liver cells via two main sporozoite antigens called circumsporozoite (CS) protein (Pancake *et al.*, 1992, Frevert *et al.*, 1993) and thrombospondin related adhesive protein (TRAP) (Müller *et al.*, 1993, Robson *et al.*, 1995) (Figures 1.2A and B). Binding is probably mediated by interactions between region II of these antigens and sulphatides (Cerami *et al.*, 1992b, Cerami *et al.*, 1992a, Pancake *et al.*, 1992, Frevert *et al.*, 1993, Müller *et al.*, 1993), and antibodies to the binding sites would provide an ideal site for host immune attack. Invasion occurs apically by invagination of the hepatocyte cell membrane to form a parasitophorous vacuole (Aikawa *et al.*, 1984), and involves the release of substances from the sporozoite rhoptries (Aikawa *et al.*, 1984). Indeed invasion probably involves a cascade of interactions between sporozoite and host molecules (Hollingdale *et al.*, 1993).

Shortly after invasion the sporozoite starts to break down to create a uninucleated trophozoite, the so called exoerythrocytic (EE) form, situated inside the parasitophorous vacuole membrane (PVM) (Aikawa *et al.*, 1984). As the trophozoite develops into the mature liver stage schizont, parasite antigens are inserted into the PVM, which forms deep invaginations (Atkinson *et al.*, 1992) particularly adjacent to the parasite nucleus (Hollingdale, 1988). Some parasite proteins are also found in the cytoplasm of the infected hepatocytes (Hollingdale, 1988, Charoenvit *et al.*, 1995). There has been just one report of the presence of parasite antigens on the surface of hepatocytes (Rénia *et al.*, 1990), suggesting that antigen presentation does not occur on the surface of the hepatocyte. Precisely how pre-erythrocytic antigens are processed and presented to T cells thus remains unclear. Many pre-erythrocytic antigens have now been characterised and the main ones are discussed below.

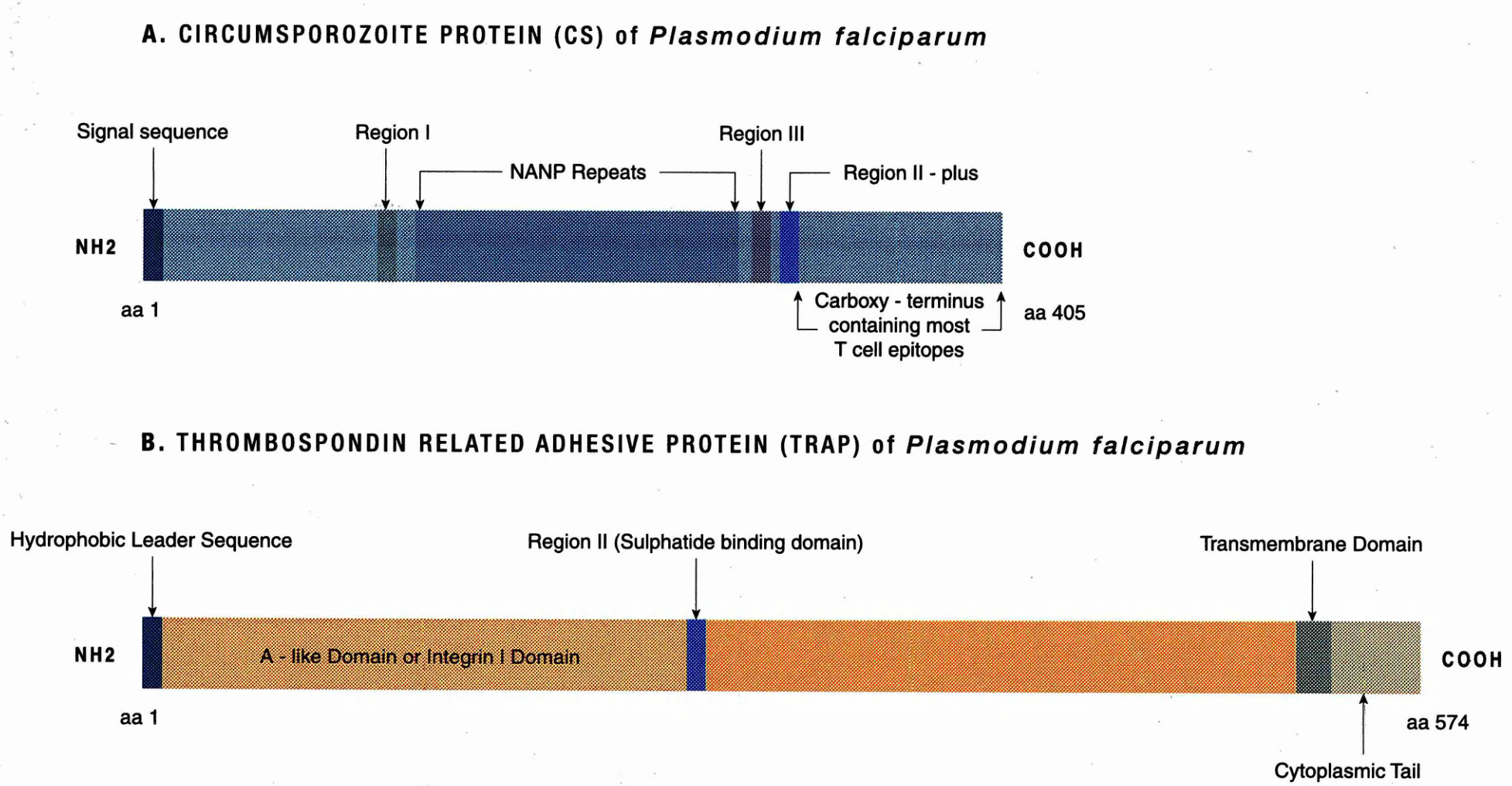
1.4.1

Circumsporozoite Protein (CS) (Figure 1.2A)

Circumsporozoite (CS) protein was the first pre-erythrocytic Ag to be identified as a target of protective immune responses (Yoshida *et al.*, 1980), and is certainly the most extensively studied. It is the most abundantly expressed Ag on the surface of sporozoites (Nussenzweig and Nussenzweig, 1985), and is also found on the micronemes (Fine *et al.*, 1984, Aikawa *et al.*, 1990). CS protein is involved in the binding to and invasion of target cells (Cerami *et al.*, 1992a, Pancake *et al.*, 1992, Frevert *et al.*, 1993), is shed on entering the hepatocyte when the sporozoite invades, and is present on the EE parasite membrane and PVM. There is no evidence for transcription, translation or expression of CS protein within hepatocytes, and in fact some data suggests the contrary (Atkinson *et al.*, 1989).

CS protein genes of the different *Plasmodium* species (*Plasmodium falciparum, P. berghei, P. cynomolgi, P. knowlesi, P. vivax, P. yoelii* etc.) encode proteins of similar structures (Figure 1.2A) but limited homology at the nucleotide and amino acid level (Santoro *et al.*, 1983). CS proteins have a hydrophobic leader sequence, followed by a central region of tandem repeats which are particular to the species.

Figure 1.2



Diagrammatic representation of the structure of CS protein and TRAP of P. falciparum (clone NF54)

Two regions, designated region I and region II, flank the amino and carboxy regions respectively of the repeats, and are highly conserved between *Plasmodium* spp. Region I contains basic amino acids and has been implicated in the process of sporozoite/hepatocyte recognition (Aley *et al.*, 1986). Region II contains the sulphatide binding motif (Cerami *et al.*, 1992b, Pancake *et al.*, 1992, Müller *et al.*, 1993, Sinnis *et al.*, 1994) that has been found in a growing number of adhesive proteins involved in cell-cell and cell-matrix interactions (Lawler and Hynes, 1986, Klar *et al.*, 1992, Leung-Hagesteijn *et al.*, 1992). The remaining carboxy-terminus of CS protein contains most of the T cell epitopes, which are discussed later (Good *et al.*, 1988d). In the case of *P. falciparum* these regions are highly polymorphic (de la Cruz *et al.*, 1987, Good *et al.*, 1988c, Good *et al.*, 1988d, de Groot *et al.*, 1989), which complicates the use of CS protein in malaria vaccines.

1.4.2

Thrombospondin Related Adhesive Protein (TRAP) (Figure 1.2B)

Thrombospondin related adhesive protein (TRAP) (Robson *et al.*, 1988), also known as sporozoite surface protein 2 (SSP2) (Rogers *et al.*, 1992), is located in micronemes and secretory organelles of sporozoites, and is released onto the sporozoite surface upon contact with target cells (Gannt *et al.*, 2000). TRAP is involved in the gliding motility of sporozoites (Sultan *et al.*, 1997), and their attachment to and entry into hepatocytes (Müller *et al.*, 1993, Robson *et al.*, 1995). It is also present at the liver stage (Aikawa *et al.*, 1990), where it is expressed for the first 4 days of infection (Rogers *et al.*, 1992). TRAP may also be expressed at low levels by erythrocytic stages of some strains of *P. falciparum* (Robson *et al.*, 1988).

The TRAP family of proteins consists of distinct domains (Figure 1.2B), although the precise structure of the molecule has yet to be determined. There is a hydrophobic leader sequence, then a conserved motif, followed by a region called the 'A domain' (or I domain, aa 48-241) which exhibits sequence similarity with a group of proteins including the integrins (Let *et al.*, 1995). Next is a sulphatide binding domain which contains the conserved peptide sequence motif (EWSPCSVTCG, aa 249-258) that is found in region II

of all CS proteins (Dame *et al.*, 1984, Arnot *et al.*, 1985, Eichinger *et al.*, 1986, Galinski *et al.*, 1987, Lal *et al.*, 1987b), and a variety of other adhesive proteins such as thrombospondin (Lawler and Hynes, 1986) and properdin (Goundis and Reid, 1988). Proteins and synthetic peptides containing this sequence bind a variety of sulphated glycoconjugates, in particular glycosaminoglycans (GAGs) such as heparin sulphate or heparin (Holt *et al.*, 1989, Sun *et al.*, 1989, Prater *et al.*, 1991, Müller *et al.*, 1993), suggesting that this motif confers specific binding properties. TRAP also contains the cell-recognition signal RGD (aa 307-309) which is characteristic of many adhesive glycoproteins and involved in cell recognition, and an IQQ motif (aa 76-78) which has been implicated in cross-linking to factor VIII. The analogous proteins in *Eimeria* spp. and *Cryptosporidium parvum* are very similar in these 2 regions, and it has been suggested that these molecules are involved in the signalling process related to invasion. These are followed by the transmembrane domain (aa 512-540 of *Pf*TRAP) and cytoplasmic tail (aa 541-574) at the carboxy-terminus.

*Pf*TRAP is polymorphic (Robson *et al.*, 1990, Robson *et al.*, 1998), although less so than CS protein. All nucleotide changes occurring outside the conserved sulphatide binding domain result in amino acid changes, and the high ratio of coding to non-coding changes implies that these mutations are a direct response to immune selection pressure (Robson *et al.*, 1990). The majority of mutations occur within the 'A domain', and few mutations are predicted to lead to structural or functional changes (Robson *et al.*, 1998). However, they could alter antigenicity and thereby provide a mechanism of immune evasion by the parasite. The same point mutations are found in parasites from different geographic locations (Robson *et al.*, 1998), which may be a reflection of structural constraints which limit the repertoire of variants found. Alternatively these mutations may have arisen at a bottleneck during evolution before spread to different parts of the world, although phylogenetic data for TRAP suggest that it is still evolving (Robson *et al.*, 1998).

Natural immunity to TRAP has not been studied in detail despite the interest in it as a vaccine candidate. The CTL epitopes identified to date are distributed in different regions

of the molecule, are all conserved, and natural responses occur at very low precursor frequencies (Aidoo *et al.*, 1995, Doolan *et al.*, 1997, Plebanski *et al.*, 1997a). No CD4 epitopes had been identified in TRAP at the time of commencing this thesis.

1.4.3

Other Pre-erythrocytic Antigens

LSA-1

LSA-1 was the second protein identified in infected hepatocytes (Guérin-Marchand *et al.*, 1987). Expression is detected throughout liver schizogeny and increases as maturation progresses, suggesting active synthesis (Fidock *et al.*, 1994b). It is present in the parasitophorous vacuole, and may adhere to merozoites (Fidock *et al.*, 1994b). The protein contains a large central repeat region that is variable in length, and is flanked by relatively invariant regions (Fidock *et al.*, 1994b, Yang *et al.*, 1995).

PfEXP-1

The mouse homologue of *P. falciparum* exported protein-1 (*Pf*EXP-1) is *P. yoelii* HEP17 (*Py*HEP17). The genes for both proteins have now been cloned, revealing striking homology between them. A mAb to *Py*HEP17 (NYLS3) recognises infected hepatocytes 6 hours after sporozoite invasion, and also recognises ring forms and trophozoites of infected erythrocytes (Charoenvit *et al.*, 1995). During the liver stage *Py*HEP17 can be found in abundance on the surface of the parasitophorous vacuole and in the cytoplasm. The mAb is able to eliminate *P. yoelii*-infected but not *P. berghei*-infected hepatocytes from *in vitro* culture (Charoenvit *et al.*, 1995). The pattern of expression of *Py*HEP17 and *Pf*EXP-1 is very similar (Sanchez *et al.*, 1994).

SALSA

SALSA was identified by screening a set of 120 DNA clones expressing pre-erythrocytic antigens against human sera that strongly recognised native proteins on the sporozoite surface and in liver stages, but did not recognise CS protein or LSA-1 (Marchand and Druilhe, 1990). SALSA is present on sporozoites and is actively synthesised and expressed in the liver stage. No homology has yet been found between SALSA and any other human or rodent *Plasmodia* spp.

STARP

The sporozoite threonine- and asparagine-rich protein (STARP), cloned by Fidock and colleagues in 1994 (Fidock *et al.*, 1994a), is present on all sporozoites of *P. falciparum* (Fidock *et al.*, 1994c) in an unusual nonhomogeneous distribution. The gene was identified using the same method as for SALSA detailed above (Marchand and Druilhe, 1990). Immunofluorescence studies support the presence of STARP at the liver stage. The 2-kb gene encodes a mosaic of multiple motifs and tandem repeats. Many repetitive and non repeat regions are conserved (Fidock *et al.*, 1994c), and DNA hybridization and probing reveals similar conserved genes for *P. yoelii* and *P. berghei*, but no homology with *P. vivax*.

LSA-3 / RESA

LSA-3 or RESA (ring-infected erythrocyte surface antigen) is an abundant 205-kDa protein expressed at sporozoite, liver and blood stages, with the same distribution as LSA-1 during the liver stage. The gene shows polymorphism in the number of repeats, but not sequence.

Heat shock proteins

Two members of the heat shock protein family have been cloned from *P. falciparum*, namely hsp70 (Bianco *et al.*, 1986, Yang *et al.*, 1987) and a glucose regulated protein GRP78 (Kumar *et al.*, 1988, Kumar and Zheng, 1992). They are expressed following invasion of hepatocytes (Kumar *et al.*, 1993), but not by sporozoites themselves. *Pf*hsp70 was localised in the nuclei and cytoplasm of EE parasites, and GRP78 was found in their nuclear membranes and endoplasmic reticulum (Kumar *et al.*, 1993). *Pf*hsp60 has now been cloned and is implicated in protective γδ T-cell responses (Tsuji *et al.*, 1994).

CSP-2

Sera and a mAb from sporozoite immunised mice recognise a 42/54-kDa antigen designated CS protein 2 (CSP-2) localised to the PVM of *P. falciparum* and *P. berghei* EE forms (Sina *et al.*, 1995). CSP-2 has not yet been cloned.

Blood Stage Antigens Expressed in Hepatocytes

Several blood stage antigens are first expressed in infected hepatocytes including *Pf*MSP-1, *Pf*EMP2 and rhoptry antigen 1. Their significance as targets of immunity at the liver stage has not been established.

1.5

ANTIBODIES & PRE-ERYTHROCYTIC IMMUNITY

1.5.1

Antibodies in Animals

Early studies of Ab mediated immunity at the pre-erythrocytic stage of infection focused on responses to CS protein. Initial observations in the *P. berghei* model of rodent malaria showed that antibodies alone against CS protein could confer protection (Yoshida *et al.*, 1980), and many groups have since demonstrated protective anti-CS Abs in the mouse (Potocnjak *et al.*, 1980, Egan *et al.*, 1987, Tam *et al.*, 1990). The immunodominant B cell epitope in CS protein recurs in tandem and is highly conserved within each *Plasmodia* species (Godson *et al.*, 1983, Dame *et al.*, 1984). However, antibodies are not an absolute requirement for protection since μ-suppressed (B cell deficient) mice immunised with irradiated sporozoites are still protected against challenge (Chen *et al.*, 1977). There is limited data suggesting that antibodies attack antigens present within infected hepatocytes. This has been shown for Abs against the heat shock proteins (Rénia *et al.*, 1990) and *Py*HEP17 (Charoenvit *et al.*, 1995). Antibodies may also develop to exoerythrocytic (EE) merozoite antigens, and these in turn may block the invasion of red blood cells after merozoites are released from hepatocytes.

1.5.2

Antibodies in Humans

Nardin and colleagues demonstrated in 1997 that naturally exposed humans have Abs against sporozoites (Nardin *et al.*, 1979). There is evidence for a protective role for anti-CS Abs in humans (Clyde *et al.*, 1975, Hoffman *et al.*, 1986, Schofield *et al.*, 1987c, Riley *et al.*, 1990), although several studies have now demonstrated that protection does not correlate with anti-CS antibody levels in individuals living in high malaria transmission regions (Riley *et al.*, 1990, Hoffman *et al.*, 1987). Antibodies to the extracellular domain of TRAP may inhibit infectivity of sporozoites (Müller *et al.*, 1993), but a more recent study suggests that this is not the case (Gannt *et al.*, 2000). Partially protective antibody responses to TRAP have been demonstrated in humans (Scarselli *et al.*, 1993), and while it

is probable that anti-TRAP Abs play a role in protection against malaria, field studies suggest that they are unlikely to induce sterile immunity.

1.5.3

Antibody Inducing Vaccines In Animals & Humans

Anti-CS Ab inducing vaccines had variable success in the murine model (Egan *et al.*, 1987, Lal *et al.*, 1987a, Zavala *et al.*, 1987), although one study showed unequivocal protection (Zavala *et al.*, 1987). Initial attempts to develop an anti-sporozoite vaccine in humans focused on inducing Ab responses to CS protein. These first-generation malaria vaccines looked promising in preliminary trials since two of four volunteers with the highest anti-CS Ab levels were completely protected (Ballou *et al.*, 1987, Herrington *et al.*, 1987), although only two out of nine individuals vaccinated were protected overall. Several studies have now demonstrated that protection does not correlate with anti-CS antibody levels in volunteers immunised against CS protein (Stoute *et al.*, 1997). In fact no vaccine has been reliably able to induce sterile immunity in humans based on the induction of anti-CS antibodies alone (Nardin and Nussenzweig, 1993, Stoute *et al.*, 1997, Good *et al.*, 1988a, Herrington *et al.*, 1991).

The protective effect of immunising with irradiated sporozoites vanishes if the sporozoites are irradiated too heavily. This suggests that sporozoites invade the hepatocytes in order to elicit protective immunity, and that immunological functions other than Ab production are involved in protection. Moreover, B cell deficient irradiated sporozoite immunised mice are able to spontaneously clear malaria parasites and resist infection (Chen *et al.*, 1977). Nevertheless, a pre-erythrocytic vaccine is likely to include Ab inducing B cell epitopes, and thus optimal Ab induction remains an important goal. As adjuvents and vaccine constructs become more immunogenic, very high Ab levels look achievable and are certain to contribute to protection. The CS protein based vaccine RTS,S/SBAS2 has now been shown to induce high anti-CS Ab levels both in naïve volunteers and naturally exposed populations (Stoute *et al.*, 1997, Doherty *et al.*, 1999).

1.6

$CD8^+$ T CELLS & PRE-ERYTHROCYTIC IMMUNITY

Precisely how antigen is processed and presented for class I recognition at the pre-erythrocytic stage of infection is not fully understood. Nearly all nucleated cells express MHC class I, so if infected can present Ag to $CD8^+$ T cells. MHC class I can be expressed on the surface of hepatocytes, although only one study has demonstrated surface expression of a liver stage antigen (Rénia *et al.*, 1990). The possible role of Kupffer cells lining the liver sinusoids, and macrophages, in processing and presentation of sporozoite derived antigens has been discussed above. Liver endothelium and circulating dendritic cells may also play a role in class I restricted antigen processing and presentation of liver stage antigens.

1.6.1

IFN-γ Mediated Cytotoxicity

The "classic" pathways of $CD8^+$ T cell mediated cytotoxicity involve either the release of perforin / granzyme, or an interaction between the target cell surface receptor called Fas (CD95) and it's ligand on the effector cell called Fas ligand (CD95L). Both pathways lead to apoptosis and target cell death (Kägi *et al.*, 1994, Lowin *et al.*, 1994). However, protection can be independent of conventional cytotoxic mechanisms. Thus, sporozoite immunisation of perforin and Fas deficient knock out (KO) mice induces protection against *P. berghei* infectious sporozoite challenge. (Renggli *et al.*, 1997). It is now believed that IFN-γ mediated cytotoxicity is the most important means of cytotoxic elimination of infected hepatocytes.

IFN-γ has been shown to inhibit the development of EE forms parasites both *in vitro* and *in vivo* in the murine and simian models (Ferreira *et al.*, 1986b, Schofield *et al.*, 1987a, Mellouk *et al.*, 1991), and the protective immunity conferred by irradiated sporozoite immunisation can be abrogated by injection of anti-IFN-γ mAbs (Maheshwari *et al.*, 1986, Schofield *et al.*, 1987c). Hepatocytes possess tens of thousands of IFN-γ receptors, and it

is assumed that IFN-γ binds to these receptors rendering the hepatocyte incapable of supporting the growth of malaria parasites (Sarkar and Gupta, 1984).

IFN-γ is thought to exert its protective effect via the induction of reactive nitrogen intermediates, in particular nitric oxide (NO). Addition of different inducible nitric oxide synthase (iNOS) inhibitors both *in vitro* and *in vivo* reverses the protection generated by attenuated sporozoites (Mellouk *et al.*, 1991, Nüssler *et al.*, 1993, Seguin *et al.*, 1994, Klotz *et al.*, 1995). Nitric oxide mediated antiplasmodial activity can be demonstrated *in vitro* in both murine and human hepatocyte cultures (Mellouk *et al.*, 1994). Mice treated with anti-IFN-γ mAb prior to sporozoite challenge failed to produce iNOS in their livers, suggesting that the induction of NOS is IFN-γ dependent (Seguin *et al.*, 1994). Inducible nitric oxide synthase expression in livers following sporozoite challenge is limited to infected hepatocytes, and depends on the persistence of irradiated parasites within the hepatocytes (Klotz *et al.*, 1995, Scheller and Azad, 1995). Mice lacking the IFN-γ receptor (IFN-γR$^{0/0}$) are not protected by a single injection of *P. yoelii* irradiated sporozoites, whereas wild type mice are protected and express high levels of iNOS mRNA in their liver (Tsuji *et al.*, 1995). However, a second injection restored protection suggesting that other mechanisms may also play a role in protection at the pre-erythrocytic stage.

Both CD4 and CD8 T cells are thought to mediate their protective effect via the production of IFN-γ. T cell responses against malaria are generally αβ T cell mediated, but γδ T cells can also have significant activity against infected hepatocytes (Tsuji *et al.*, 1994, Langhorne *et al.*, 1995, Rzepczyk *et al.*, 1997). Natural killer (NK) cells might also have a protective effect (Doolan and Hoffman, 1999), although their precise role remains unclear. Despite the evidence suggesting a protective role for IFN-γ mediated T cell responses, few studies of T cell immunity at the pre-erythrocytic stage have assessed for IFN-γ production and protection. One study found a protective effect for cultured IFN-γ responses against LSA-1 derived peptides (Luty *et al.*, 1999), but no studies have assessed for protective efficacy in the field using assays that detect rapid IFN-γ release by ELISPOT assay.

1.6.2

$CD8^+$ T Cells in Animals

Early experiments suggested that pre-erythrocytic immunity is dependent on $CD8^+$ T cells. Treatment of sporozoite-immune mice with anti-CD8 Ab abrogates their immunity (Schofield *et al.*, 1987c, Weiss *et al.*, 1988), and protective immunity was lost following *in vivo* depletion of $CD8^+$ T cells, but not $CD4^+$ T cells (Schofield *et al.*, 1987c, Weiss *et al.*, 1988). CS protein specific $CD8^+$ T cell clones have since been shown to mediate protective immunity in mouse models of malaria, even to a single CTL epitope (Romero *et al.*, 1989, Weiss *et al.*, 1992). Mice immunised with irradiated *P. berghei* sporozoites and challenged with live sporozoites produce parasite-specific $CD8^+$ T cell-dependent inflammatory infiltrates in their livers (Hoffman *et al.*, 1989a). Spleen cells from these mice eliminate infected hepatocytes from hepatocyte cultures that are free of Kupffer cells, in a class I MHC-dependent and species specific manner (Hoffman *et al.*, 1989a, Hoffman *et al.*, 1990) suggesting that *Plasmodium* antigens on the surface of infected hepatocytes are recognised by T cells leading to killing.

Adoptive transfer experiments demonstrate that CTL against a single 9mer CTL epitope in CS protein can confer protection against sporozoite challenge (Romero *et al.*, 1989, Romero *et al.*, 1990, Rodrigues *et al.*, 1991, Weiss *et al.*, 1992). Adoptive transfer of a $CD8^+$ CTL clone against *Py*SSP2/TRAP protected 100% of mice against challenge (Khusmith *et al.*, 1994). Transfer of $CD8^+$ T cell clones 3 hours after sporozoite challenge, at a time when the parasites have already entered hepatocytes, can still prevent development of blood stage infection (Rodrigues *et al.*, 1991, Khusmith *et al.*, 1994). There is also experimental evidence for a protective role for CTL directed against other pre-erythrocytic antigens including LSA-1 and *Py*HEP17 (EXP1) (Hollingdale *et al.*, 1990, Atkinson *et al.*, 1992, Charoenvit *et al.*, 1995).

1.6.3

$CD8^+$ T Cells in Humans

Sporozoite immunised human volunteers have $CD8^+$ T cell-dependent, peptide specific cytolytic activity against a CTL epitope region of CS protein which is homologous to a CTL epitope 1st identified in mice (Malik *et al.*, 1991). Malaria exposed Australians, and lifelong exposed Kenyans, were subsequently shown to have peptide specific $CD8^+$ T cell-dependent CTL against a similar epitope region of CS (Doolan *et al.*, 1991, Sedegah *et al.*, 1992). Several HLA restricted $CD8^+$ T cell epitopes have since been identified in CS, TRAP, LSA-1 and STARP in naturally exposed donors from The Gambia (Hill *et al.*, 1992b, Aidoo *et al.*, 1995) and Burkina Faso (Blum Tirouvanziam *et al.*, 1995). Eleven novel HLA-A2 restricted epitopes in SSP2 / TRAP were identified in studies of volunteers immunised with irradiated sporozoites (Wizel *et al.*, 1995a, Wizel *et al.*, 1995b). These volunteers had direct cytotoxic activity without the need for *in vitro* re-stimulation, thus demonstrating the presence of circulating activated CTLs against a *P. falciparum* antigen for the first time.

1.6.4

CTL Inducing Pre-erythrocytic Vaccines in Animals

CTL responses are HLA class I restricted, therefore sufficient antigens or epitopes would need to be incorporated in a vaccine to cover the range of HLA types present in the population to be vaccinated. Indeed, different vaccines may be required for different ethnic groups where HLA types vary widely. Stimulation of adequate CTL responses is the next logistic problem. Strategies conventionally employed, such as the use of attenuated organisms are not practical in the case of malaria, and the current focus of attention is on molecular techniques.

A considerable number of $CD8^+$ T cell inducing vaccines have been tested in the mouse model of malaria including oral recombinant salmonella (Sadoff *et al.*, 1988), recombinant vaccinia (Satchidanandam *et al.*, 1991) and pseudorabies (Sedegah *et al.*, 1990) based vaccine constructs expressing CS protein. Most early studies using these constructs failed

to induce protective CTL, although oral recombinant salmonella achieved 50-75% protection against *P. berghei* challenge (Sadoff *et al.*, 1988). Immunisation of mice with plasmid DNA encoding either CS protein, PyHEP17 or TRAP (SSP2), induced 50-90% protection in certain mouse strains, but not in others in a number of separate studies (Sedegah *et al.*, 1994b, Doolan *et al.*, 1996, Wang *et al.*, 1998). There are concerns about the long term safety of DNA vaccines (Hedstrom *et al.*, 1990, Mor *et al.*, 1997), although trials in animal models show a good safety record, and human trials have commenced.

Vaccines consisting of combinations of antigens generally give better protection than constructs based upon one antigen alone. For example, the combination of transfected P815 cells expressing *P. yoelii* CS protein and *Py*SSP2 / TRAP gave full protection (Khusmith *et al.*, 1991), and a combination of 2 DNA vaccine constructs encoding *Py*CS and *Py*HEP17 was more effective than either construct alone (Doolan *et al.*, 1996). Priming mice with a recombinant influenza virus expressing a *P. yoelii* CS $CD8^+$ T cell epitope, and boosting with recombinant vaccinia expressing the whole of *P. yoelii* CS, increased protection to the 60% level (Li *et al.*, 1993). Regimes involving a DNA prime and MVA boost (Schneider *et al.*, 1998), or Ty particle prime and MVA boost (Plebanski *et al.*, 1998), have since induced complete protection. Protection in the latter study was highly correlated with the induction of high numbers of IFN-γ secreting $CD8^+$ T cells detected by ELISPOT assay.

1.6.5

CTL Inducing Pre-erythrocytic Vaccines in Humans

Over the last 5 years a number of CTL inducing vaccines for use in humans have been constructed. These include an oral recombinant salmonella expressing CS protein (Gonzalez *et al.*, 1994), hepatitis B particles carrying part of CS protein (Gordon *et al.*, 1995), and attenuated vaccinia expressing several liver stage antigens (Ockenhouse *et al.*, 1998). Each of these constructs induced $CD8^+$ CTL in certain donors, although only the hepatitis B construct showed significant protection in 2 out of 8 volunteers who received a certain formulation (Gordon *et al.*, 1995).

There is optimism that DNA vaccination will be a suitable means of inducing protective CTL (Hoffman *et al.*, 1998), and clinical trials are ongoing. It is more likely however that methods designed to optimise the DNA vaccination will ultimately be employed such as prime-boost strategies which have proved so effective in murine models (Plebanski *et al.*, 1998, Schneider *et al.*, 1998, Sedegah *et al.*, 1998). Modified vaccinia Ankara (MVA) is a highly attenuated strain of vaccinia which was used extensively in humans as past of the smallpox eradication campaign, with no serious adverse effects (Mayr *et al.*, 1978). This seems an excellent candidate for use in prime-boost strategies, although many other CTL inducing vaccine constructs might be considered including Ty particles (Weber *et al.*, 1995) and adenovirus (Fender *et al.*, 1997).

A widely applicable CTL inducing vaccine construct would require epitopes or antigens recognised by individuals with a variety of HLA types (Doolan *et al.*, 1996). Use of the *P. falciparum* CTL epitopes reported to date together as a string could provide coverage of over 75% of European and West African individuals (Aidoo *et al.*, 1995). Problems such as antigenic polymorphism (Doolan *et al.*, 1992) and altered peptide ligand antagonism (APL) (Gilbert *et al.*, 1998) must also be overcome in the choice of epitopes and antigens (see below).

1.7

$CD4^+$ T CELLS & PRE-ERYTHROCYTIC IMMUNITY

For more than a decade pre-erythrocytic vaccine development focused on the induction of high levels of Abs and $CD8^+$ T cells. There is now increasing evidence that $CD4^+$ T cells also play an important protective role, which may be as potent as CD8 mediated protection. The precise nature of CD4 T cell induced protection is not understood. MHC class II is mainly present on the surface of APCs, including Kupffer cells, but needs to be induced. It may also be induced and expressed by hepatocytes. IFN-γ can induce class II expression by endothelial cells, and this may play a role in class II mediated immunity in malaria. $CD4^+$ T cells that are remote from the infected hepatocyte might be activated by

sporozoite derived antigens, or antigens secreted by hepatocytes. These activated $CD4^+$ T cells might release cytokines such as IFN-γ and kill the parasite infected hepatocytes.

1.7.1

$CD4^+$ T Cells in Animals

The first $CD4^+$ T cell responses to a pre-erythrocytic antigen were described for the *P. berghei* model in 1977 (Spitalny *et al.*, 1977). Murine studies subsequently showed that there is an immunodominant T cell antigenic region on CS protein, denoted Th2R (aa 326-343) which falls in a polymorphic domain (Good *et al.*, 1987). Adoptive transfer of a $CD4^+$ cytolytic T cell clone recognising a sporozoite antigen (not CS protein) from sporozoite immunised mice confers protection against sporozoite challenge (Tsuji *et al.*, 1990). $CD4^+$ T cells directed against *P. yoelii* CS protein have been shown to recognise CS protein peptides on the surface of infected hepatocytes, eliminate infected hepatocytes in culture, and confer protection in adoptive transfer experiments (Rénia *et al.*, 1991, Rénia *et al.*, 1993, Takita-Sonoda *et al.*, 1996). The majority of studies demonstrating $CD4^+$ T cell activity at the pre-erythrocytic stage of infection in mice have been to CS protein, although a more recent study demonstrated $CD4^+$ T cell activity against a *P. yoelii* 17-kilodalton hepatocyte erythrocyte protein (Charoenvit *et al.*, 1999).

1.7.2

$CD4^+$ T Cells in Humans

The seminal study mapping human CD4 T cell epitopes in CS protein was performed by Good and colleagues using PBMC from naturally exposed West Africans (Good *et al.*, 1988d). They tested for lymphoproliferative responses to 29 overlapping 20mer synthetic peptides spanning the whole of CS protein. Three immunodominant domains denoted Th1R (aa 301-320), Th2R (aa 326-345) and Th3R (aa 361-380) were found within the highly polymorphic carboxy-terminus of CS protein. One of these domains overlapped the mouse T cell helper epitope region Th2R (aa 326-343), identified in studies as a helper epitope for *P. falciparum* anti-CS Ab production (Good *et al.*, 1987). Indeed, the human

$CD4^+$ T cell epitope regions in the carboxy-terminus of CS seem to correspond with those found in the murine model (Dontfraid *et al.*, 1988).

Sinigaglia and colleagues identified another immunodominant region in CS protein denoted CS.T3 (aa 379-398) (Sinigaglia *et al.*, 1988a). They selected 3 peptide sequences (CS.T1, CS.T2, CS.T3) predicted to contain T cell epitopes, and tested for lymphoproliferative responses in 16 malaria exposed (Ivory Coast) and 6 malaria naïve individuals. Nine of the 16 malaria exposed individuals responded to CS.T3, and all 3 peptides induced responses in some donors. T cell clones to CS.T3 were derived from one of the responders, and the cells were found to be $CD4^+CD8^-$, and required specific class II haplotypes for effective antigen presentation (Sinigaglia *et al.*, 1988a). Three of the CS.T3 clones recognised *Pf*CS protein purified from sporozoites. Four of the 6 malaria naïve donors also responded to at least one peptide. This might be explained by the presence of cross-reactive epitopes with other microorganisms arising at these regions of the CS protein.

Field studies in Kenyans showed that lymphoproliferative responses to two T cell epitope regions in the carboxy-terminus of CS protein (aa 361-380; aa 371-390), the former being the Th3R epitope, significantly correlate with resistance to re-infection with malaria (Hoffman *et al.*, 1989b). However the numbers were very small in the non-parasitaemic group, and the evidence for protection is not compelling. Riley and colleagues assessed lymphoproliferative responses to 3 polymorphic strains of the Th2R and Th3R regions of CS protein in 391 Gambian children aged 3 to 8 years. They were unable to confirm the protective effect of the Th3R epitope observed by Hoffman and colleagues, but did find a protective association between lymphoproliferation to this epitope at the beginning of the transmission season and subsequent susceptibility to infection among those children with high anti-CS Ab levels (Riley *et al.*, 1990). Thus, seropositive children who did not become infected had a higher mean proliferative response to the Th3R region than seropositive children who did become infected.

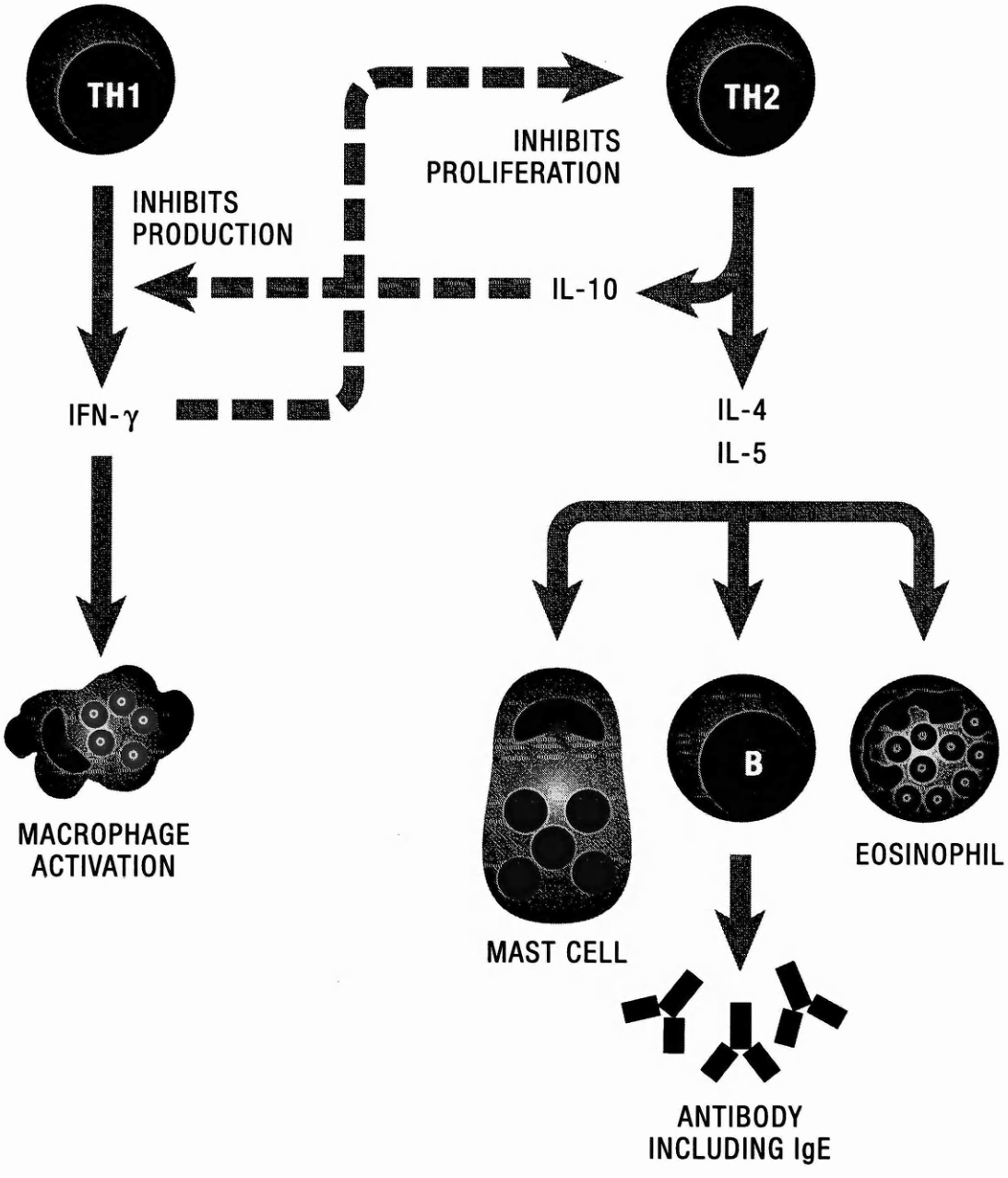
$CD4^+$ CS-specific CTL have also been demonstrated in sporozoite immunised humans (Moreno *et al.*, 1991, Moreno *et al.*, 1993). These studies identified DR1-, DR4-, DR7- and DR9-restricted distinct T cell epitopes within aa 326-345 of *P. falciparum* CS protein, all of which have been cloned. $CD4^+$ T cell clones from these studies specifically lysed autologous B cells pulsed with a synthetic peptide representing the C-terminal region of CS protein (Moreno *et al.*, 1991). CD4 epitope regions have now been identified in several other pre-erythrocytic antigens including LSA-1 (Fidock *et al.*, 1994b, Krzych *et al.*, 1995, Yang *et al.*, 1995), LSA-3 (BenMohammed *et al.*, 1997), and SALSA (Bottius *et al.*, 1996). No CD4 T cell epitopes have been identified within TRAP, despite the fact that this is currently considered a good vaccine candidate antigen.

1.7.3

Th1 and Th2 $CD4^+$ T Cell Dichotomy

$CD4^+$ T cells can be broadly divided into 2 functional groups named T helper type 1 (Th1) and T helper type 2 (Th2) (Mosmann and Coffman, 1987, Mosmann and Coffman, 1989), although other subgroups are now known to exist (Groux *et al.*, 1997). These 2 groups are characterised by distinctive patterns of cytokine release, and different immunological functions (Figure 1.3). Th1 $CD4^+$ T cells produce cytokines such as IFN-γ, IL-2 and TGFβ; whereas Th2 $CD4^+$ T cells are typified by the production of IL-4 and IL-5, with other cytokines such as IL-10 and IL-13 also commonly produced. Th1 cytokines activate cytotoxic, inflammatory and delayed hypersensitivity reactions. By contrast Th2 cells encourage Ab production, particularly IgE, and are associated with regulation of Ab and allergic responses. Cytokines from Th1 cells inhibit Th2 cells, and vice versa, and thus most CD4 T cell responses settle into a Th1 or Th2 type pattern. This Th1/Th2 dichotomy depends on a number of factors including antigen dose, local cytokine profile, and activation of co-stimulatory molecules.

Figure 1.3



CD4 T cells can be broadly divided into Th1 and Th2 types, each with distinct effector functions. Th1 type cytokines include IFN-γ, TNF-β, IL-3 and IL-12. Th2 type cytokines include IL-4, IL-5, IL-6, IL-10 and IL-13.

The protective $CD4^+$ T cell response at the pre-erythrocytic stage of malaria infection is thought to be mediated by the Th1 type cytokine IFN-γ. The evidence that IFN-γ is responsible for T cell mediated cytotoxicity in protection at the pre-erythrocytic stage of malaria infection was discussed earlier in relation to $CD8^+$ T cells (section 1.6.1). The same mechanisms are thought to be responsible for $CD4^+$ T cell mediated protection against malaria. Thus, IFN-γ release by Th1 type $CD4^+$ T cells leads to induction of reactive nitrogen intermediates, in particular nitric oxide (NO), via the nitric oxide synthase pathway, which in turn kill the infected hepatocytes. However, there is a fine balance between the production of a protective inflammatory response, and that response being destructive (Hirunpetcharat *et al.*, 1999).

Administration of recombinant IL-12, another Th1 type cytokine, to mice and rhesus monkeys provides 100% protection against sporozoite challenge with *P. yoelii* and *P. cynomolgi* respectively (Sedegah *et al.*, 1994a, Hoffman *et al.*, 1997). The protection in mice is entirely eliminated with mAb to IFN-γ, and is eliminated in 50% of mice by administration of an iNOS inhibitor. It is thought that IL-12 induces T cells and NK cells to produce IFN-γ, which induces infected hepatocytes to produce the NO that kills developing parasites (Sedegah *et al.*, 1994a).

There is evidence that Th2 type responses play a regulatory role at the blood stage of malaria infection (Troye-Blomberg *et al.*, 1990, Stevenson and Tam, 1993, Taylor-Robinson *et al.*, 1993, al-Yaman *et al.*, 1997), but few studies have assessed for the production of Th2 type cytokines at the pre-erythrocytic stage of malaria infection in animals (Rénia *et al.*, 1993, Takita-Sonoda *et al.*, 1996) or humans (Doolan *et al.*, 1994, Kurtis *et al.*, 1999). The inhibitory effects of the Th2 type cytokine IL-6 on intrahepatic development of human and murine parasites have been reported (Pied *et al.*, 1991), and high levels of peptide specific IL-6 production to CS protein is observed in naturally exposed donors from Papua New Guinea (Doolan *et al.*, 1994). An inverse correlation between IFN-γ and IL-6 production was noted in this study, which is typical of the Th1/Th2 dichotomy discussed above.

Th2 type $CD4^+$ T cell clones can eliminate liver stage parasites *in vitro* and *in vivo* in the mouse model of malaria (Rénia *et al.*, 1993). Th2 type $CD4^+$ T cell clones can also provide protection against murine *P. c. chabaudi* infection in adoptive transfer experiments, probably via the enhancement of IgG1 production (Taylor-Robinson *et al.*, 1993). Th2 type T cells may also have a regulatory role by downregulating the Th1 type IFN-γ mediated inflammatory response. A role for IL-10 producing cells at the pre-erythrocytic stage of infection remains controversial . IL-10 downregulates Th1 type responses (Mosmann and Moore, 1991), and thus while it may prevent the induction of protective responses, it may also control the pro-inflammatory response leading to host tissue damage (Kurtis *et al.*, 1999, Li *et al.*, 1999). Thus, although Th2 type $CD4^+$ T cell responses have been described at the pre-erythrocytic stage, the main bias is towards a protective Th1 type response characterised by the production of IFN-γ. Indeed, the contribution of Th2 type responses to pre-erythrocytic stage immunity in humans remains poorly characterised.

1.7.4

$CD4^+$ T Cell Inducing Vaccines in Animals

BALB/C mice immunised with a multiple antigen peptide known to contain a protective $CD4^+$ T cell epitope from *P. berghei* CS protein were protected against subsequent sporozoite challenge (Migliorini *et al.*, 1993). Several further studies have similarly shown $CD4^+$ T cell peptide specific protection following immunisation with CS derived peptides (Takita-Sonoda *et al.*, 1996, Charoenvit *et al.*, 1999). A vaccine based upon an 18 amino acid peptide from PySSP2/TRAP in an anionic polymer adjuvent provides 100% protection in A/J mice, but little protection in 2 other strains (Wang *et al.*, 1996). The immunity is eliminated by *in vivo* addition of anti-CD4 Abs, but not anti-CD8 Abs. Microscopic analysis of the livers indicate that immunity is directed against the infected hepatocytes and eliminated by treatment with anti-IFN-γ. *In vitro* studies have shown that this peptide activates only a Th1 type T cell response, and that protection is better than

that found with any univalent vaccine designed to induce protective $CD8^+$ T cell responses.

1.7.5

$CD4^+$ T Cell Inducing Vaccines in Humans

A CS protein based vaccine called RTS,S protected 6 out of 7 malaria naïve volunteers against subsequent malaria challenge (Stoute *et al.*, 1997). This vaccine consists of a hybrid of the central NANP repeat region, most of the carboxy-terminus of CS (containing the main T cell epitope regions) fused to hepatitis B surface Ag in a complex adjuvent mixture (SBAS4). The anti-CS Ab levels induced were very high in most cases but did not correlate with protection in these donors. RTS,S induced high levels of IFN-γ secreting CS-specific $CD4^+$ T cells, and no $CD8^+$ T cell responses were detectable (Stoute *et al.*, 1997, Lalvani *et al.*, 1999). The $CD4^+$ T cell responses induced by RTS,S are thought to play a role in the protection seen in vaccinees. Phase I field trials are now complete (Doherty *et al.*, 1999), and Phase II field trials are currently underway in The Gambia. This is the first pre-erythrocytic stage vaccine to go to field trials in a malaria endemic population.

Thus, vaccine development has mainly focused on the induction of $CD8^+$ T cell responses to pre-erythrocytic stage antigens, despite clear evidence for a protective role for $CD4^+$ T cells. CD4 T cell induction is now regarded as a useful component in malaria vaccine design. Several $CD4^+$ T cell epitope regions have been identified within CS protein, but they mainly fall within highly polymorphic regions of the antigen. Any new $CD4^+$ T cell epitopes identified within other more conserved pre-erythrocytic antigens such as TRAP might therefore be useful components of a future malaria vaccine, particularly those that are conserved.

1.8

OTHER CYTOKINES & PRE-ERYTHROCYTIC IMMUNITY

In addition to the protective role for the Th1 type cytokine IFN-γ, and a putative role for Th2 type responses mediated by IL-4, IL-5 and IL-10, there is evidence to suggest that

other cytokines are also protective (Del Giudice *et al.*, 1990, Nüssler *et al.*, 1991a). The inhibitory effects of IL-1 on intrahepatic development of human and murine parasites have been reported (Mellouk *et al.*, 1987). Tumour necrosis factor (TNF) inhibits the development of *P. berghei in vitro* in a hepatoma cell line (Schofield *et al.*, 1987b), but not *P. yoelii*-infected hepatocytes (Mellouk *et al.*, 1991, Nüssler *et al.*, 1991b). In co-cultures of hepatocytes and nonparenchymal cells, TNF has been shown to inhibit parasites by inducing nonparenchymal cells to release IL-6 (Nüssler *et al.*, 1991a, Nüssler *et al.*, 1991b).

The concept of an anti-toxic vaccine is based upon the observation that individuals who are frequently exposed to malaria suffer few symptoms, but remain susceptible to infection (Playfair *et al.*, 1994). Many immune processes are probably at play here, including the acquisition of Abs to tumour necrosis factor (TNF-)-inducing parasite toxins (Bate and Kwiatkowski, 1994). Parasite antigens probably induce a variety of cytokines, and the critical matter is the fine balance between a protective response, and one that is harmful. It is important not to suppress the host's natural cytokine response to infection to an extent that gives increased susceptibility to the parasite (Kwiatkowski, 1995).

1.9

HLA & PRE-ERYTHROCYTIC IMMUNITY

Several studies suggest that malaria plays a role in the maintenance of MHC polymorphism. Thus, different MHC alleles are thought to provide varying degrees of protection against malaria due to restriction of T cell recognition of malaria antigens to certain HLA types. Hill and colleagues demonstrated that the HLA class I antigen HLA-B*53, and the HLA class II haplotype DRB1*1302-DQB1*0501, are associated with reduced susceptibility to severe malaria (Hill *et al.*, 1991). Both of these HLA types are more frequent in West Africans than other racial groups, suggesting that natural selection by malaria contributed to this elevated frequency.

P. falciparum contains thousands of potential T cell epitopes, each with varying HLA restriction patterns. The fact that certain HLA types are protective suggests that many

responses are probably of limited protective value. In an attempt to identify HLA-B53 restricted T cell epitopes that might mediate the protective effect of this HLA allele, 60 putative HLA-B53 binding peptides were synthesised from 4 pre-erythrocytic antigens: namely CS protein, TRAP, sporozoite hepatocyte binding antigen (SHEBA) and liver stage antigen 1 (LSA-1). Only one HLA-B53 restricted CTL epitope was identified in malaria immune Gambians, within the pre-erythrocytic antigen LSA-1, suggesting that this epitope may be of protective significance (Hill *et al.*, 1992b).

HLA types vary widely between the different populations of the world, and there is considerable HLA variation between different African ethnic groups (Hill *et al.*, 1992a). Despite this, the effect of this HLA variation on the pattern of T cell epitope recognition for pre-erythrocytic vaccine candidate antigens has not been investigated for different African populations. Clearly this is important in terms of malaria vaccines designed to be widely effective among different ethnic groups.

1.10
MALARIA IMMUNOSUPPRESSION

Widespread nonresponsiveness to malaria antigens has been a consistent feature in field studies of T cell responses in naturally exposed populations. Thus, despite a lifetime of exposure, T cells specific for both variant and conserved pre-erythrocytic malaria antigens are not found consistently in healthy aparasitaemic individuals (Good *et al.*, 1988d, Riley *et al.*, 1988b, Quakyi *et al.*, 1989, Troye-Blomberg *et al.*, 1989, Eposito *et al.*, 1992, Doolan *et al.*, 1993, Doolan *et al.*, 1994, Plebanski *et al.*, 1997a). T cell responses are also generally suppressed during the course of malaria infection (Ho *et al.*, 1986, Theander *et al.*, 1986, Riley *et al.*, 1988a, Chemtai and Okelo, 1989, Hviid *et al.*, 1991b). The precise mechanisms of such immunological non-responsiveness remain unclear, but a variety of mechanisms have been proposed.

1.10.1

T Cell Tolerance and Anergy

T cell tolerance is classically described as the intrathymic deletion of self reactive T cell clones during the neonatal period (Burnet, 1959). It has been proposed that T cell tolerance induction might explain generalised non-responsiveness to malaria antigens. There is considerable homology between malaria and human Ags (McLaughlin *et al.*, 1987), and these homologous regions eg the thrombospondin like domain in CS protein (Lawler and Hynes, 1986) and TRAP (Robson *et al.*, 1988), are poorly immunogenic. Active tolerance induction may occur during the neonatal period, and T cells that respond to those foreign proteins or peptides seen at this stage when immunity is developing may be deleted. Indeed, T cell tolerance was induced in neonatal mice by immunising with CS protein specific T and B cell epitopes (Pombo *et al.*, 1988). Thus children may be born with a degree of T cell tolerance to malaria proteins in endemic regions of the world.

There is evidence that post thymic mechanisms can lead to tolerance in peripheral T lymphocytes. Post thymic tolerance induction may depend on a number of factors including the antigen dose (Ferber *et al.*, 1994), and the route of exposure and frequency of dosing (Aichele *et al.*, 1995). If antigen is not presented with the correct co-stimulatory molecules, or by professional antigen presenting cells, this may also lead to induction of tolerant T cells (Lamb *et al.*, 1983). Whether this should be described as true tolerance or anergy is debatable, since the mechanism is more one of functional silencing of T cells, rather than clonal deletion. Therefore anergy might seem a more appropriate description.

The presence of small amounts of antigen on the surface of hepatocytes can lead to T cell anergy to that antigen (Ferber *et al.*, 1994), an effect that may be because hepatocytes lack the necessary co-stimulatory molecules to activate T cells successfully. Similarly, expression of small doses of pre-erythrocytic antigenic epitopes on the surface of infected hepatocytes in naturally exposed donors may be one mechanism whereby malaria specific anergy might be induced. Repeated stimulation by normal APC can lead to T cell anergy in previously activated T cells (Suzuki *et al.*, 1988). Since malaria exposure is continuous and

repeated throughout life, this mechanism may also lead to malaria specific peripheral tolerance induction. It has been shown that T cell responsiveness to malaria antigens can be restored in PBMC from healthy individuals *in vitro* by the addition of IL-2 (Mshana *et al.*, 1990), and IL-2 is known to reverse T cell anergy in certain cases (Essery *et al.*, 1988). It is feasible that the IL-2 is acting on anergic cells in these donors. The contribution of tolerance and / or anergy to the T cell non-responsiveness to pre-erythrocytic malaria antigens is still not understood.

1.10.2

'Original Antigenic Sin'

The concept of 'original antigenic sin' describes a process whereby epitope sharing between pathogens leads to one pathogen inducing responses before the other pathogen has been experienced. When exposure to the second pathogen occurs, a skewed repertoire of responses results due to prior exposure to the cross-reactive epitope from the first organism. It has been proposed that this mechanism may play a role in the lack of T cell reactivity in malaria, despite lifelong exposure (Good *et al.*, 1993). Malaria antigens contain regions homologous to other organisms, and exposure to such non-malaria organisms may lead to priming of T cell responses which are cross-reactive with malaria epitopes. Indeed many malaria naïve adults have T cells specific for malaria parasites (Zevering *et al.*, 1992, Good *et al.*, 1993). These cross-reactive T cells may preferentially expand following exposure to malaria sporozoites, and thus skew the repertoire of the T cell response. This may in turn divert the immune response towards these common epitope regions which may not be protective, and away from responses to protective T cell epitopes within the antigen.

1.10.3

Immunosuppressive T Cells Subsets

T cell responsiveness can be restored in PBMC from healthy individuals *in vitro* by the removal of supposedly immunosuppressive $CD8^+$ T cells (Riley *et al.*, 1989, Mshana *et al.*, 1990, Theander *et al.*, 1993), which suggests an immunosuppressive role by activated

T cells. Indeed, seasonal variations in peripheral T cell function have been directly demonstrated to be due to differences in the suppressive capacity of $CD8^+$ T cells (Theander *et al.*, 1993), although the precise nature of these putative suppressor cells has yet to be established.

1.10.4

T Cell Redistribution

Neither IL-2 nor CD8 T cell removal restores T cell responsiveness in malaria patients, suggesting another mechanism for the immunosuppression (Ho *et al.*, 1986, Ho *et al.*, 1988). In fact these patients exhibit decreased suppressor cell function (Gilbreath *et al.*, 1983), and several studies have demonstrated malaria-associated lymphopaenia, with reduced $CD3^+$ and $CD4^+$ T cells (Ho *et al.*, 1986). A decrease in cells expressing high levels of the endothelial adhesion factor LFA-1 (lymphocyte function associated antigen type 1) is also a feature of malaria (Hviid *et al.*, 1991a). It is thought that T cells become redistributed during malaria episodes, presumably to sites of endothelial activation.

1.10.5

Antigenic Polymorphism

Current strains of *P. falciparum* are estimated to have arisen from a single ancestral strain some 25-60 thousand years ago, depending upon the nucleotide substitution rate (Rich *et al.*, 1998). This has allowed the parasite time to develop numerous strategies for avoiding the host's natural defences. The immune selection pressure afforded by malaria is apparent when one considers that it is responsible for maintaining a variety of genetic disorders such as sickle cell, thalassaemia and G6PD deficiency within the gene pool, simply because the heterozygous state is protective against *P. falciparum* (Allison, 1954, Flint *et al.*, 1986, Ruwende *et al.*, 1995). Exactly how $CD4^+$ and $CD8^+$ T cells exert their selective pressure on the malaria parasite leading to the generation of polymorphism remains unclear.

At the pre-erythrocytic stage of infection the most highly expressed antigen, CS protein, has developed numerous polymorphisms in the dominant T cell epitope regions (de la

Cruz *et al.*, 1988b, Good *et al.*, 1988c, Good *et al.*, 1988d, de Groot *et al.*, 1989). Most nucleotide changes lead to amino acid changes, suggesting there is a powerful immune selection pressure operating at these sites. The highest level of variation within CS is found in two immunodominant proliferation-inducing CD4 T cell epitope regions within the carboxy-terminus of CS, denoted Th2R (aa 326-347) and Th3R (aa 361-380) (Good *et al.*, 1988d), where up to 14 different variants of Th2R occur naturally (Lockyer *et al.*, 1989, Doolan *et al.*, 1992, Shi *et al.*, 1992), nine of which co-exist in The Gambia where many of the studies in this thesis were based (Conway *et al.*, 1991, Lockyer *et al.*, 1989). CD8 T cell epitopes, denoted Tc2R (aa 327-335) and Tc3R (aa 369-375), also exist within these regions and exhibit a high level of polymorphism (Doolan *et al.*, 1991, Malik *et al.*, 1991, Shi *et al.*, 1992, Aidoo *et al.*, 1995).

Field studies demonstrate that responses to polymorphic epitope regions within CS protein are generally strain specific (de la Cruz *et al.*, 1988a, de la Cruz *et al.*, 1989, Udhayakumar *et al.*, 1994, Zevering *et al.*, 1994), although one study in Gambians shows a high degree of cross-recognition of 3 strains (de Groot *et al.*, 1989). Thus, polymorphism probably provides an immune escape strategy whereby certain strains are not recognised despite previous parasite exposure. Such antigenic diversity presents a major obstacle for malaria vaccine development. One strategy to overcome this is to focus on conserved regions of antigens, although possibly the reason such regions remain conserved is that they are nonprotective. The pre-erythrocytic antigens TRAP, LSA-1 and STARP are more conserved, perhaps making them more attractive vaccine candidates than CS protein (Fidock *et al.*, 1994b, Fidock *et al.*, 1994c, Robson *et al.*, 1998).

1.10.6

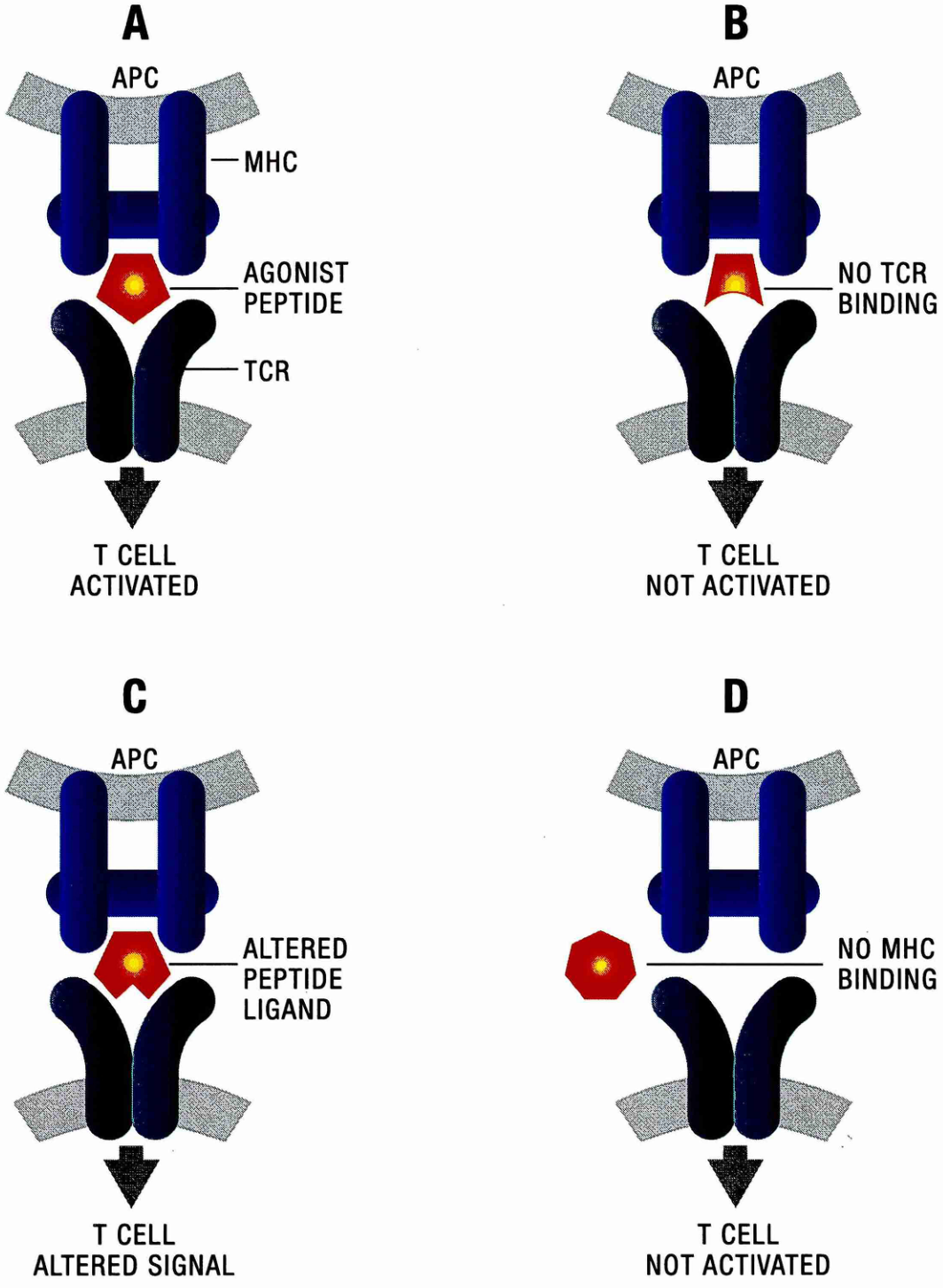
Altered Peptide Ligand Antagonism / Immune Interference

There is good evidence that natural selection by malaria plays a role in maintaining the MHC polymorphism seen in malaria endemic regions of the world (Hill *et al.*, 1991, Hill *et al.*, 1992b). The precise mechanism for this is not fully understood, although the process known as altered peptide ligand (APL) antagonism may play a role. This is the process

whereby the simultaneous presence of a variant antagonistic epitope delivers an altered signal to the responding T lymphocyte leading to either decreased or absent responsiveness to the agonist peptide (Figure 1.4). Studies of CTL responses to HIV and hepatitis B show that certain naturally occurring variants of epitopes within these organisms specifically down-regulate the CTL response by APL antagonism (Bertoletti *et al.*, 1994, Klenerman *et al.*, 1994). Gilbert and colleagues found that two HLA-B35 restricted CD8 T cell epitopes in CS protein, named cp26 and cp29, can lead to impairment of CTL activity by this process (Gilbert *et al.*, 1998). These epitopes vary by just one amino acid residue at position 2, and are able to interfere with the activation of memory cells to one another. It was found that cp26 and cp29 occur together more frequently in naturally exposed children than would be expected by chance. The authors conclude that cohabitation of cp26 and cp29 could result from the strains facilitating one another's survival through mutual antagonism of CTL to the other variant at the liver stage of infection.

Mathematical modelling of population dynamics of closely related variant epitopes in *P. falciparum* malaria have demonstrated that a necessary component for the persistence of new variants is an ability to avoid specific *de novo* responses (Gupta and Hill, 1995). It has been shown that APL antagonism for the CTL variants cp26 and cp29 of CS protein can operate at the level of T cell priming, and prevent the generation of peptide specific T cells from naïve precursors when presented on the same antigen presenting cell (APC) (Plebanski *et al.*, 1997b). This process has been termed "immune interference", and in malaria exposed populations could lead to a lifelong inability to respond to a particular parasite strain, and thus exposed but functionally naïve hosts. Such immune interference has been demonstrated *in vivo* in a mouse model of malaria where co-immunisation with an agonist / antagonist combination of peptide immunogens led to a substantial decrease in the primed response to the agonist (Plebanski *et al.*, 1999). This immune evasion strategy is more powerful than the selection of epitopes which fail to bind MHC or to cross-react, since it prevents the emergence of alternative target epitopes (Gilbert *et al.*, 1998, Davenport, 1995).

Figure 1.4



Altered peptide ligands of T cell epitopes prevent correct T cell activation (as in A) by 3 main mechanisms: preventing T cell recognition through the MHC receptor (B), altered signal through the T cell receptor (C), or failure to bind to the MHC molecule (D).

Interference with T cell priming by APL antagonism generates defective effector T cells, capable of proliferation in response to antigen, but not of killing, or of producing potentially protective liver stage cytokines like IFN-γ or TNFα (Plebanski *et al.*, 1999).

The phenomena of APL acting at the effector stage, and causing immune interference of T cell priming, are very important considerations in terms of malaria vaccine design. Vaccines containing APL may antagonise protective T cell responses to naturally occurring variants, rather than boost a protective response. Immune interference may prevent the priming of protective immune responses in malaria naïve vaccinees. The existence of altered peptide ligands has not been studied for the CD4 epitope regions of CS protein, or indeed for CD4 T cell responses to any infectious pathogen. It is important to establish whether such mechanisms are at play for any antigen intended for use in malaria vaccines.

CHAPTER 2

METHODS

2.1

ANTIBODY ELISA

Antibody ELISAs were performed in The Gambia by technical staff in the malaria laboratory. The antibody responses to the tetrapeptide (NANP) repeat of CS were measured using a specific ELISA with plate adsorbed R32LR antigen. A standard reference serum of known concentration (provided by the Walter Reed Army Institute of Research, Washington DC, USA) was used on every plate as a positive control (Gordon *et al.*, 1995). Microwells were coated with 50 µl of R32LR at 0.1mg/ml in PBS 0.2% boiled casein, and were incubated overnight at room temperature (RT). After emptying, wells were blocked with 0.5% boiled casein / 1% Tween in phosphate buffered saline (PBS) (Sigma, Dorset, UK) for 1 hour at RT. Wells were aspirated and 50 µl of the appropriate serum dilution was added per well. Assays were conducted in triplicate, with blank wells and standard reference positive and negative sera on each plate. After 2 hours incubation at RT plates were washed 4X with PBS-1% Tween, and 50µl of anti-human conjugated peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. Plates were incubated for 1 hour at RT, washed 4X with PBS-1% Tween, and 100 µl of ABTS substrate solution was added per well. After a further 1 hour incubation at RT, 10 µl of 20% SDS (Sigma, Dorset, UK) was added to stop the reaction, and absorbance read at 414 nm. Titres were expressed in comparison to the standard reference serum.

2.2

BINDING ASSAYS

HLA-DR binding assays for the 9 Th2R variants of CS were carried out by Drs Colin Gelder and Keith Hart. They were performed blind to all cellular results using the principal of competitive binding with a promiscuously binding invariant chain [li-(96-114)] biotinylated CLIP peptide (I*) as described (Davenport *et al.*, 1995a, Davenport *et al.*,

1995b). Test peptides in DMSO (Sigma, UK) were serially diluted 1:10 in pH5 buffer (0.02 M 2-N-morpholinoethaqne-sulphonic acid in 0.1 M NaCl and 0.02% sodium azide) and incubated at 37°C for 24 hr with competitor peptide I* (0.1 mg) and purified HLA class II protein (0.15 mg). Solutions were neutralised with Tris-HCl (pH 7.5) before transfer to 96 well BSA-blocked immunoplates (Nunc, UK) pre-coated with L-243 Ab (anti-HLA class II) (Sigma, UK). After 2 hr, plates were washed 3X with PBS 0.1% Tween 20 (PBS-T) (Sigma, UK) and 3X with PBS. Plates were developed with avidin-horseradish peroxidase (ExtrAvidin; Sigma, St Louis, MO, USA) / biotinylated anti-avidin mAb (Sigma, UK) and developed with o-phenylene diamine (0.4 mg / ml) in phosphate citrate buffer. The reaction was monitored every 5 min at 495 nm and was terminated with 12.5% sulphuric acid. Peak pre-equilibrium values were used to calculate the concentration of unlabelled peptide required to inhibit 50% binding of *I (-(Ymax*X / (IC_{50} + X) + background) using GraphPad Prism 2.0. Binding assays were repeated in triplicate at least twice and the mean calculated.

2.3

CLONING BY LIMITING DILUTION

PBMC from donor D57 (HLA-DR15,4;-DQ6, 8;-DP52,53) were resuspended at $4x10^6$/ml in RPMI/10% NHS supplemented with 2% penicillin/streptomycin and 1% L-glutamine (RN10) and cultured at 37°C, 5% CO2 with 25μg/ml V6 peptide. On day 7, blast T cells were isolated by density gradient interphase on Percoll (Sigma, UK) (30%-40% dilution in RPMI) after 20 min centrifugation at 600xg, were pelleted, resuspended in media with 10u/ml human purified IL-2 (Lymphocult T-HP, Biotest AG, Dreieich, Germany), and cultured for 7 days at 37°C, 5% CO2. The blast cell line was shown to be V6 specific and HLA-DR15 restricted in IFN-γ ELISPOT assays utilising a panel of class II matched and mismatched PBMC as presenters (data not shown). The cultured isolated blast cells were pelleted and resuspended with autologous irradiated (2000 rads) PBMC at a ratio of 1:50 effectors:presenters in RN10 media supplemented with 10u/ml IL-2. Blast cell dilutions of 1 cell, 10 cells and 100 cells per 20μl of media were made, and 20μl of these preparations were plated onto terasaki plates (Costar, UK), and incubated at 37°C for 1 week. 10μl of

RN10 media with 10u/ml IL-2 was added to each well, and plates incubated for a further 7 days. The cells in the terasaki plates were then scored visually under the microscope, and positive wells were transferred to 96 well round bottomed plates (Beckton Dickinson, France). These cells were then restimulated using a 3 way mixed lymphocyte reaction (MLR) with irradiated (2000 rads) PBMC in 10u/ml IL-2 and 1μg/ml PHA (Wellcome Pharmaceuticals, UK), and placed in an incubator at 37°C / 5% CO2. 10u/ml IL-2 was added every 5 days and cells were split when necessary.

Six clones were isolated from 600 initial wells (C1-C6). Clone C1 failed to survive for re-testing, and was excluded from the study. The T cell receptor (TCR) β-chain from clones C3 and C4 was sequenced by Dr. G. Gillespie in Oxford confirming for each the existence of a single dominant β-chain TCR product (data not shown). T cell clones were confirmed to be CD4 positive by FACScan analysis (data not shown).

2.4

CONTROL ANTIGENS

The following control antigens were used where indicated throughout the text:

Purified protein derivative from *Mycobacterium bovis* (PPD) (Serumstatens Institut, Denmark) was used as the positive control for IFN-γ *ex-vivo* ELISPOT assays and lymphoproliferation assays; the mitogen phytohaemagglutinin (PHA) (Wellcome Pharmaceuticals, UK) was used as a positive control in IL-4 and IL-10 ELISPOT assays; tetanus toxoid (TT) (Evans Biomedical, UK) was used as an additional positive control for selected ELISPOT assays. PPD, TT and PHA were reconstituted in sterile media, and were added to wells to give the correct final concentration. Media alone was added to the negative control wells to give the background response.

2.5

DNA EXTRACTION

All DNA extractions were performed by myself, Mr. P. Akinwunmi (in The Gambia), and Mr. K. Odhiambo and Mr. E. Sheu (in Kenya) using Qiagen QIAmp Blood Kits (Qiagen,

Crawley, UK). All reactions were carried out at room temperature (unless otherwise stated) and all reagents / collection tubes were provided in the kits. 200µl of heparinised whole blood or Ficoll red blood cell pellet (see PBMC preparation) was placed in a 1.5 ml microfuge tube, and 25µl of QIAGEN Protease stock solution and 200µl Buffer AL added to the sample. The pellet was vortexed for 15 seconds, and incubated for 10 minutes at 70°C. 210 µl of ethanol (96-100%) was added to the sample which was then vortexed for 15 seconds. A QIAmp spin column was placed in a 2ml collection tube. The above mixture was applied, the cap closed, and centrifuged at 6000xg for 1 minute. The QIAmp spin column was placed in a clean 2ml collection tube, and the tube containing the filtrate discarded. The spin column was opened, 500 µl of Buffer AW added, and spun at 6000xg for 1 minute. The spin column was placed in a clean 2ml collection tube, and the filtrate discarded. Another 500 µl of Buffer AW was added to the spin column, and spun for 3 minutes in a microfuge at full speed. The spin column was placed in a clean 1.5ml microfuge tube, and the tube containing the filtrate discarded. The DNA was eluted with 200 µl of Buffer AE preheated to 70°C, and the tube incubated at RT for 1 minute, then centrifuged at 6000xg for 1 minute. Eluted DNA was stored at -20°C until required for analysis.

2.6

DONORS

The studies in Africa were carried out at 2 centres, namely the MRC Research Unit in Fajara, The Gambia, and the Wellcome Trust Research Laboratories in Kilifi, Kenya. Different cohorts of donors were used for different studies, and these are detailed in the relevant chapters.

2.6.1

The Gambia, West Africa

The studies in chapters 3, 4 and 5 involved the use of PBMC from Gambian donors. Maps and location of donors are detailed in the text.

2.6.2

Kilifi District, Kenya, East Africa

The studies in chapters 5 and 6 were performed using PBMC from Kenyan donors recruited from Kilifi District, Kenya. Maps and location of donors are given in the text.

2.6.3

Malaria Naïve Donors

Healthy adult donors who had never been exposed to malaria were recruited from the staff at the John Radcliffe Hospital in Oxford, UK. All were aged 21-50 years, and a travel history was taken to ensure no previous risk of malaria exposure. All donors were fit and well at the time of blood sampling. A further 15 healthy adults were also recruited in Oxford for other studies regardless of previous exposure to malaria.

2.7

ELISPOT ASSAYS

2.7.1

ELISPOT Coating Plates

All ELISPOT assays were carried out using 96 well MAIP S4510 0.45μm plates coated with a hydrophobic high protein binding Immobilon-P membrane / polyvinylidene (PVDF) (Millipore, Watford, UK). ELISPOT kits supplied by Mabtech (Mabtech, Nacka, Sweden) were used for all ELISPOT assays. The plates were coated initially with either monoclonal anti-human IFN-γ Ab (1-D1K), anti-human IL-4 (IL4-I) or IL-10 (9-D7) according to the cytokine to be assayed. The coating mAb was diluted to a concentration of 15 μg/ml in sterile PBS (Sigma, Dorset, UK), and 50μl added to each well of the 96 well plate. Plates were incubated for 3 hours at room temperature, or overnight at 4°C. The coating mAb was then flicked off, and plates washed 5X with sterile PBS (Sigma, Dorset, UK). The plates were then blocked by adding 100μl RPMI-1640/10% FCS (R10) to each well, and incubating for 1 hour at room temperature. This blocking media was then flicked off and the plate was ready for adding the cells to be assayed for cytokine production.

2.7.2

ELISPOT *Ex-vivo* PBMC / Lines / Clones

All cells were resuspended in RPMI 1640 supplemented with 5% pre-selected batch tested normal human serum (NHS) and 2% penicillin/streptomycin, 1% L-glutamine (RN5 media). The cell concentrations used were $8x10^6$/ml for freshly isolated PBMC, $1x10^5$/ml for T cell lines, and $1x10^4$/ml for T cell clones, following preliminary titrations (not shown). Cells were applied at 50µl per well, and the peptide or control antigen, and APCs when indicated, were added to each well to make a final volume of 100µl. Peptides and PPD were used at a final concentration of 25µg/ml following preliminary titrations (see below), unless otherwise stated. $8x10^4$ cells were used in the PHA positive control test wells for the IL-4 and IL-10 *ex-vivo* ELISPOT assays, and PHA was used at 1µg/ml. Irradiated (2000 rads) APCs (see PBMC Methods) were added to ELISPOT wells at an APC:responder cell ratio of 1:4 in assays using PBMC and positively selected T cells; 100:1 for clones and 10:1 for cell lines. Incubation times were optimised following preliminary studies in Oxford (data not shown). Plates were incubated for 16 hours at 37°C, 5% CO2 for the IFN-γ assay, and 24 hours for IL4 and IL10 assays.

2.7.3

ELISPOT Cultured Cells

The culture medium for all cultured ELISPOT assays was α-MEM (Life Technologies, UK) with 10% batch tested NHS, supplemented with 2% penicillin/streptomycin and 1% L-glutamine (MEM10). $1x10^6$ cells in 1ml MEM10 were placed in separate wells of a 48 well flat bottomed tissue culture plate, and peptides / peptide pools were added to give a final concentration of 25µg/ml per peptide. Plates were placed in an incubator at 37°C, 5% CO2, and 10u/ml IL-2 (Lymphocult T, Biotest AG, Dreieich, Germany) was added on days 5 and 10. On day 14 the PBMC were washed once in RPMI, and resuspended at $1x10^6$/ml. They were then tested at 100µl/well in 96 well MAIP ELISPOT plates ($1x10^5$ cells) in standard 16 hour IFN-γ ELISPOT assays against the test peptides and negative control.

2.7.4

ELISPOT Peptide Titrations

Preliminary titration assays with CS and TRAP derived peptides were performed to assess the optimal range for T cell epitope mapping. This was established to be within the range 10-100μg/ml. I chose a working concentration of 25μg/ml for TRAP peptides and PPD since at this concentration all positive ELISPOT responses will be detected, although peak responses generally occurred at 50-100μg/ml. Phytohaemagglutinin was used as the positive control for IL-4 and IL-10 assays at 1μg/ml to ensure that the detection reagents were working. PPD and PHA responses were unaffected by the addition of selected CS protein and TRAP peptides used in the studies, over a range of concentrations from 10 to 100μg/ml, thus confirming their lack of toxicity (data not shown).

2.7.5

ELISPOT Antagonism Assays

PBMC in media in 96 well ELISPOT plates were first pre-pulsed with index peptide at a suboptimal concentration of 25μg/ml, and incubated at 37°C, 5% CO2 for 1 hour. The antagonistic peptide was then added directly to the individual test wells at a concentration of 50-100μg/ml. The plate was left to incubate at 37°C, 5% CO2 as for the standard *ex-vivo* ELISPOT assay according to the cytokine coating the plate (IFN-γ, IL-4 or IL-10).

2.7.6

ELISPOT Inhibitory mAb Assays

Anti-TGFβ (Genzyme, Cambridge, MA) and anti-IL-10 (Genzyme, Cambridge, MA) were applied to the *ex-vivo* ELISPOT wells at 10ng/ml for both mAbs. Where reversal of inhibition was not observed (for anti-TGFβ) the dose was increased to 100ng/ml. The mAbs were added to the ELISPOT wells 1 hour before the putative antagonistic peptides, at the same time as the index peptide.

2.7.7

ELISPOT Developing Plates

The ELISPOT plates were removed from the incubator after the required incubation time (see above) and the cells flicked off. The plates were then washed 4 times with PBS containing 0.05% Tween 20 (PBS-T) (Sigma) to remove adherent cells, and then twice with PBS alone. A second biotinylated monoclonal Ab to either IFN-γ (7-B6-1-Biotin, Mabtech, Nacka, Sweden), IL-4 (IL4-II-Biotin, Mabtech), or IL-10 (12G8-Biotin, Mabtech) was diluted to 1μg/ml with sterile PBS. 50μl of the mAb solution was applied to each well, and plates were incubated for a minimum of 2 hours at room temperature. The plates were then washed again as above (4X PBS-T, 2X PBS). The conjugation enzyme streptavidin alkaline phosphatase (Mabtech, Nacka, Sweden) was diluted to a concentration of 1μg/ml in sterile PBS, and 50μl was applied to each ELISPOT well. Plates were incubated for a further 1 hour at room temperature, and washed again as above (4X PBS-T, 2X PBS). Cytokine secreting cells were visualised by the addition of the chromogenic precipitable alkaline phosphatase substrate 5-bromo 4-chloro 3-indolyl phospho/nitro blue tetrazolim (BCIP/NPT) (Bio Rad, Hemel Hempstead, UK). This was prepared by the addition of 200μl of 20X Development Buffer, 50μl of Substrate A and 50μl of Substrate B, added in that order to 4.8 mls of sterile distilled water (Sigma, UK). 50μl of this solution was added to each ELISPOT well, and allowed to incubate at room temperature until dark purple spots emerged (typically 10-20 minutes). The reaction was then halted by washing plates under running tap water, and plates were left to dry in air for a few hours.

Wells were scored with the aid of a dissection microscope at x20 magnification, for the number of cells producing IFN-γ, according to the number of purple spots or spot forming units (SFUs) per well. Three observers scored every positive well in order to increase our confidence in this subjective scoring system. The results were expressed as SFUs / 10^6 PBMC added. In certain cases results were confirmed using the AID Elispot reader (Autoimmun Diagnostika GmbH, Strasberg, Germany), and scored using the AID Elispot software version 2.0 (Autoimmun Diagnostika). A peptide specific response was scored

positive if the antigen stimulated response was statistically above the background (unstimulated) response, with 95% confidence using a statistical table (see Section 2.16.1 for details).

2.8

FLUORESCENCE ACTIVATED CELL SORTER (FACS)

2.8.1

FACS Cell Surface Marker Staining

Cells for FACScan analysis were stained with the relevant monoclonal Abs (fluorochrome conjugated CD3, CD4, CD8, CD14, CD22, CD38) derived from a number of sources (DAKO, Denmark / Becton Dickinson, USA / Serotec, UK) according to the protocol recommended for each individual antibody. Generally, cells were washed in 1% NHS in PBS/0.1% (w/v) sodium azide (PBS-NHS1az), pelleted and incubated with the mAb on ice for 20-30 minutes. Cells were then washed 2x with PBS-NHS1az, and resuspended in isoton buffer (Becton Dickinson) for analysis. When necessary cells were fixed with 200µl PBS/0.4% (w/v) paraformaldehyde (see below).

2.8.2

FACS Intracellular IL-10 Staining

Donor D57 $CD4^+$ T cell line ($2x10^6$ cells) derived from Percoll isolated blast cells (see Cloning Methods) was stimulated in culture with HLA-DR15 matched irradiated (2000 rads) PBMC ($2x10^6$ cells) either alone, with V6 at 20µg/ml, or with V6 at 20µg/ml first and V5 at 40µg/ml added 1 hour later. These cells were then incubated in 1ml flat bottomed wells (Becton Dickinson, France) for 18 hours at 5% CO2, 37°C, and 2µM monesin (Biosource International, USA) was then added to the cultures which were incubated for a further 6 hours. Cells were first stained with the appropriate anti-cell fluorescent labelled surface markers and fixed according to the protocol above. The fixed cells were permeabilised in PBS-NHS1az with 0.1% (w/v) saponin. Anti-IL-10 RPE-conjugated mAb (Biosource International, USA) was then added to the cells which were incubated on ice for

30 minutes. Cells were then washed twice in the permeabilisation buffer and suspended in a required volume of isoton buffer (Becton Dickinson) for FACScan analysis.

2.8.3

FACS Data Collection and Analysis

FACScanning and analysis was performed by myself, M. Plebanski (my supervisor), A. Vargas and V. Appay in Oxford. FACScanning was performed using the FACSCalibur Cell Sorter machine (Becton Dickinson, USA). Data was collected and analysed using CELLQuest software (Becton Dickinson, UK).

2.9

HLA CLASS II PHOTOTYPING

2.9.1

Polymerase Chain Reaction

HLA class II phototyping was performed by myself, and Drs C. Gelder and K. Hart according to the published protocol (Bunce *et al.*, 1995). Genomic DNA for class II HLA typing was amplified in 96 well thermowell V plates (Costar, High Wycombe, UK) using the Phoenix thermocycler (Teche, Cambridge, UK). The primer mixes and amplification primers were provided by Drs C. Gelder and K. Hart, according to the published sequences and specificity's (Bunce *et al.*, 1995). All amplification primers were designed to give a melting point of 58-62°C. PCR reaction mixtures consisted of 67mM Tris Base pH8.8, 16.6mM ammonium sulphate, 2mM magnesium chloride, 0.01% Tween 20, 200μM of dNTP, 1-4μM of each allele-specific primer, 0.1μM of DRB1 control primers, 0.1-0.01μg DNA, and 0.1875 units Taq Polymerase (Advanced Biotechnology, London, UK). The final volume of all PCR reactions was 13μl, consisting of 8μl of buffer, DNA and enzyme mixture and 5μl of primer mix (containing allele and control primers in distilled water). 10μl of mineral oil was overlayed on this.

Trays were sealed with thermowell sealer (Costar, UK). The cycling parameters for 13μl reactions in rapid-cycling PCR machines were as follows: 1 minute 96°C, 5 cycles of 25

seconds at 96°C, 45 seconds at 70°C, 45 seconds at 72°C, 21 cycles of 25 seconds at 96°C, 50 seconds at 65°C, 45 seconds at 72°C, 4 cycles of 25 seconds at 96°C, 60 seconds at 55°C, 120 seconds at 72°C.

2.9.2

Gel Electrophoresis

PCR products were electrophoresed in 2% agarose gels containing 0.5µg/ml ethidium bromide after the addition of 10µl of loading buffer consisting of 0.05% Orange G, 30% v/v glycerol, and 0.5x TBE buffer (89mM Tris base, 89mM boric acid, 2mM EDTA, pH8.0). Flowgen gel tanks (Flowgen, Sittingbourne, UK) allowed multiple loading of reactions with a multichannel pipette. Gels were run for 30-50 minutes at 15V/cm in 0.5x TBE buffer, were visualised using UV illumination, and scored according to phototype pattern.

2.10

MAGNETIC BEAD CELL DEPLETION / SELECTION

2.10.1

Reagents

Monoclonal Abs

Mouse anti-human CD45RO, CD45RA and CD45RB (all from Serotec, Oxford, UK) were used at a concentration of 1:100 with PBMC at $1x10^7$/ml in RPMI-1640/10% FCS (R10) medium (see below). Mouse anti-human CD38 (Serotec, UK) was also used at 1:100 dilution, and added to cells at $1x10^7$/ml in R10. Cells for CCR7 depletion were incubated in rat anti-human CCR7 mAb (rat IgG2a clone 3D12 provided by Dr. R. Förster, Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany) at 100µl mAb per $5x10^6$ PBMC in 100µl PBS/3% FCS.

Dynabeads

$CD4^+$ and $CD8^+$ cell depletions of PBMC were performed using anti-human M-450 CD4 (T helper) and M-450 CD8 (T cytotoxic) magnetic Dynabeads respectively (Dynal, Bromborough, UK). Pre-washed goat anti-mouse Dynabeads (Dynal, Oslo, Norway) were used to deplete cells coated with mouse anti-human mAbs (CD45RO, CD45RA, CD45RB,

CD38). Washed sheep anti-rat Dynabeads (Dynal, Norway) were used to deplete the rat anti-human CCR7 coated PBMC. $CD4^+$ cell selections from PBMC were performed using CD4 Positive Isolation Kits (Dynal, UK). For the positive selection of $CD38^+$ cells I used CELLection^{TM} Pan Mouse IgG Kit (Dynal, UK).

2.10.2

Washing Dynabeads

Dynabeads were first washed by resuspending in the vial and transferring the required amount (approximately 5 beads per cell to be depleted) into a sterile test tube, and placing the tube on a Dynal Magnetic Particle Concentrator (MPC) magnet (Dynal, Oslo, Norway). The fluid was removed by pipette, the tube removed from the magnet, and the beads resuspended in 1ml of PBS/2% FCS. The tube was re-applied to the MPC magnet, and the washing procedure with PBS/2% FCS repeated twice. Beads were resuspended to the original volume in PBS/2% FCS after washing.

2.10.3

Cell Depletion Protocols

$CD4^+$ and $CD8^+$ Cells

$CD4^+$ and $CD8^+$ cell depletions of PBMC were performed using anti-human CD4 and CD8 magnetic Dynabeads (see above) (Dynal, UK) respectively as follows. PBMC for depletion were resuspended in PBS/2% FCS at 1-5x10^7 cells/ml, and cooled to 4°C. Dynabeads were added (5 per estimated cell to be depleted) and cells incubated at 4°C for 30 minutes with gentle rotation. The rosetted cells were then placed on an MPC magnet for 2-3 minutes, and the depleted supernatant transferred to a fresh tube. Cells were recounted following a further 2 rounds of depletion on the magnet.

$CD45RO^+$, $CD45RA^+$, $CD45RB^+$, $CCR7^+$ and $CD38^+$ Cells

Depletion of PBMC expressing CD45RO, CD45RA, CD45RB, CCR7 and CD38 were performed using the same basic protocol. Thus, the relevant mAbs were added to the cell preparations at the required concentrations (see above), and incubated at 4°C for 30 minutes. Cells were then washed 3X in media, and pre-washed Dynabeads were added to

the mixture (5 per cell to be depleted). Cells were incubated with the Dynabeads for 20-30 minutes with gentle rotation at 4°C, and the rosetted cells were removed by magnetic depletion on an MPC magnet as above. Cells were washed 3X with R10, counted and set up in the relevant assays.

FACScan analysis of depleted and purified cells (see above) performed for selected donors confirmed that depletions and purifcations were 90-95% effective. 'Mock depletions' were also performed as additional controls for selected donors whereby either the mAb alone, or the beads alone, were added to PBMC in parallel assays to ensure that they were not responsible for any effects seen.

2.10.4

Positive Cell Selection

$CD4^+$ Cells

$CD4^+$ cell selections from PBMC were performed using CD4 Positive Isolation Kits (Dynal, UK). The Dynabeads were washed in exactly the same way as detailed above. PBMC for depletion were resuspended in PBS/2% FCS at 1-5x10^7 cells/ml, and cooled to 4°C. Dynabeads were added (5 per estimated cell to be depleted) and cells incubated at 4°C for 20 minutes with gentle rotation. The rosetted cells were then collected by placing on an MPC magnet for 2-3 minutes, and the supernatant was discarded. The bead bound isolated cells were then washed 4X with PBS/2% FCS, and resuspended in RPMI 1640/1% FCS (R1) at 1x10^8 cells/ml. One unit (10µl) of DETACHaBEAD was then added per 100µl of cell suspension. Cells were incubated for 45-60 minutes at room temperature with gentle mixing, then placed on an MPC magnet for 2-3 minutes. The supernatant contains the released positively selected cells, and these were transferred by pipette to a fresh tube. The beads were then washed 2X in medium and supernatant collected to obtain residual cells. Detached cells were washed 3X in R1, counted and resuspended for use in assays.

$CD38^+$ Cells

For the positive selection of $CD38^+$ cells I used CELLection™ Pan Mouse IgG Kit (Dynal, UK). The Dynabeads were washed according to the above procedure. Beads were

then coated with mouse anti-human CD38 mAb (Serotec, UK) by adding 1μg mAb (1μl of 1mg/ml) to every 100μl washed beads (0.25μg mAb / 10^7 beads), and incubating for 30 minutes at RT with gentle rotation. Beads were then placed on a Dynal MPC magnet for 2-3 minutes, and the fluid pipetted off. The tube was removed from the magnet, and resuspended in 1ml PBS/0.1% BSA. This washing procedure was performed 3X, and the mAb coated beads then resuspended in their original volume with PBS/0.1% BSA.

The PBMC were resuspended in RPMI/1% FCS at 10-40x10^6/ml and cooled to 4°C. The precoated beads were resuspended and the desired volume (5 per cell to be depleted) were transferred to a sterile tube, placed on a magnet, and the buffer removed. The PBMC suspension was then applied and the coated beads and PBMC mixed by light whirlmixing. Cells were then incubated for 15 minutes at 4°C by gentle rotation on a mixer. The tube was then placed on a magnet for 2-3 minutes, and the supernatant pipetted and discarded. The tube was removed from the magnet, and resuspended in 0.5-1ml RPMI/1% FCS, placed back on a magnet and after 2-3 minutes the supernatant removed. This washing step was repeated 3X. The rosetted cells were then resuspended in 200μl RPMI/1% FCS medium pre-warmed to 37°C, and 4μl of DNase solution (provided) added per 10^7-10^8 Dynabeads used. The tube was then incubated for 15 minutes at RT with gentle mixing, the rosetted cells flushed through a pipette and placed on a Dynal magnet for 2-3 minutes. The released cells in the supernatant were pipetted into a fresh tube containing 200μl RPMI/10% FCS. The residual cells were obtained by flushing the beads 5X through a narrow tipped pipette, placing on a magnet, and transferring the suspension to the other released cells. Cells were then counted and resuspended in appropriate media for the assay.

2.11

MALARIA BLOOD FILMS

Thick and thin malaria blood films were prepared for most donors from Kenya and The Gambia who took part in the studies. In all cases they were performed by the local laboratory staff. For thin film preparation a small drop of blood was placed on a clean glass slide, and a second slide used to pull the drop across the slide and create a smear. 2-3 drops

of blood were used for thick films, which were spread with the corner of a second slide to a thickness whereby print could be read through the film. Films were fixed in 100% methanol, dried and immersed for 10 minutes in a standard Giemsa stain (10% working solution, pH 7.2). They were then rinsed under tap water, dried and read under a light microscope.

2.12

PBMC PREPARATION

2.12.1

PBMC Isolation from Whole Blood

Heparinised whole blood was taken from donors after obtaining informed consent. This was transferred to 15ml or 50ml sterile Falcon tubes (Beckton Dickinson, France) according to the volume taken. Tubes were centrifuged at 600xg for 5 minutes, and an aliquot of plasma taken and frozen for future use. The blood was then layered onto an equal volume of Ficoll Lymphoprep^{TM} (Nycomed, Oslo, Norway) in 15ml or 50ml Falcon tubes, and centrifuged at 800xg for 20 minutes (without brake). The peripheral blood mononuclear cell (PBMC) fraction was collected from the interphase by pastette pipette into fresh sterile Falcon tubes. An aliquot of the Ficoll neutrophil fraction was frozen for DNA extraction at a later date (see DNA extraction). The PBMC were washed three times in RPMI, and counted under a microscope using 0.05% Trypan Blue solution in PBS (Sigma, UK) using a disposable counting chamber (Fast-Read 10^{TM}, Immune Systems Ltd., Paignton, UK). The PBMC were then resuspended at the appropriate cell concentration, in the media required for the assay to be performed (e.g., RN10, MEM10).

2.12.2

PBMC Red Blood Cell Lysis

In certain cases the PBMC isolated on Ficoll contained numerous red blood cells, which can interfere with the ELISPOT assay. In such cases a red blood cell lysis step was performed as follows. 10mls ACK lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1 mM Na_2EDTA, Ph 7.2) was added to the PBMC pellet, and incubated at room temperature for

5 minutes. 25mls RPMI was then added and the sample underlayed with 2mls sterile FCS. This was then spun at 600xg for 10 minutes, and the supernatant containing lysed red cells and red cell membranes discarded. The pellet was then flicked and washed twice more with RPMI, and the lysed PBMC were then ready for resuspending in media for the cellular assay.

2.12.3

PBMC Preparation of Adherent Cells

Adherent cells, for use as APCs in certain assays, were prepared from freshly isolated PBMC. The PBMC were resuspended at $5x10^6$/ml in sterile RPMI-1640/10% FCS (R10) and 4mls placed in separate wells of a 6 well flat bottomed tissue culture plate (Beckton Dickinson, France). They were incubated at 37°C, 5% CO2 for $1^1/_2$-2 hours, and the non-adherent cells removed gently by pipette. The petri dish was then washed gently with R10 medium, and further non-adherent cells removed. The adherent cells were then scraped off the dish using a cell scraper (Beckton Dickinson, UK), and resuspended in R10. The adherent cells were then irradiated to 2000 rads, counted and resuspended at the desired concentration, in the appropriate media, according to the assay for which they were required.

2.12.4

PBMC Freezing

Typically 5-30x10^6 PBMC were frozen in a single 1.5ml cryotube vial (Nalgene, UK). The isolated PBMC were centrifuged to form a cell pellet, and resuspended in 250µl of neat FCS (Sigma, UK), and left on ice for 30 minutes. Cells were then transferred into pre-prepared 1.5ml cryotube vials containing 250µl chilled 20% DMSO in FCS. The cryotubes were placed in a styrofoam container and transferred immediately to a -70°C freezer. The styrofoam allows for a gradual drop in temperature while cells are freezing at -70°C. The cryotubes were transferred to liquid nitrogen tanks for long term storage after 2 days at -70°C. The final volume of frozen PBMC was therefore 500µl in 10% DMSO/FCS. An identical method was used to freeze T cell clones and lines.

2.12.5

PBMC Thawing

The frozen PBMC were removed from liquid nitrogen tanks and thawed in a 37°C water bath. As soon as the frozen cells had thawed they were transferred to a sterile 15ml Falcon tube (Beckton Dickinson, France). RPMI/10% FCS (R10) media, that had been pre-warmed to 37°C, was added slowly to the thawed cells at a rate of 2mls/minute until 6mls, and then topped up to 10mls. The tube was centrifuged at 600xg for 5 minutes, and the pellet suspended in prewarmed R10, and centrifuged again at 600xg for 5 minutes. This step was repeated once more, after which the cells were counted using a disposable counting chamber (ISL, UK) and trypan blue (Sigma, UK) (as for freshly isolated PBMC). Generally 70-90% of frozen cells were recovered using this technique. The same method was used to thaw frozen clones and lines.

2.12.6

PBMC EBV Transformation for B Cell Lines

2-4x10^6 PBMC were centrifuged to a pellet in 15ml Falcon tube and resuspended in 500µl of EBV supernatant, and incubated at 37°C for 1 hour. The Epstein-Barr virus (EBV) supernatant was from an EBV infected marmoset cell line B95-8, which was provided by Dr. P. Thornton Everton in Oxford. The cells were washed in RPMI-1640 to remove excess virus, and resuspended in RPMI/10% FCS (R10) media with 1µg/ml cyclosporin A (Sigma, UK) at a concentration of 1x10^6 cells/ml. The infected cells were plated into 24 well tissue culture plates (Beckton Dickinson, UK), incubated at 37°C, and monitored for transformation (usually after 7 days). Every 7 days, half the medium of the infected cells was changed with fresh R10 with 2µg/ml cyclosporin A.

2.12.7

PBMC T Cell Purification

T cell purification was performed using glass bead human T cell immunocolumns (Cytovax Biotech. Inc., Alberta, Canada) according to the manufacturer's instructions. Columns were

washed with 20mls sterile PBS. Column reagent was reconstituted in 1ml sterile PBS, applied to the column, and left for 1 hour at room temperature. The column was then washed again with 20mls sterile PBS. The PBMC (maximum $1.5x10^8$/ml) were resuspended in 1ml, and applied to the column. The column was flushed through with sterile PBS, and the purifed T cells collected and counted for use in cellular assays.

2.13

PEPTIDE BINDING PREDICTION PROGRAMME

A computer programme was used which predicts the binding efficacy of peptides along the length of an antigen to particular HLA molecules (Davenport *et al.*, 1995a). This programme was derived from pool sequence data from eluted peptides purified from HLA class II proteins. This method can be used to predict likely T cell epitopes within a given protein that will bind to the HLA type in question. All known sequences for *Plasmodium falciparum* thrombospondin related adhesive protein (TRAP) were provided by Dr. K. Robson. These were scanned through the prediction programme to identify sequences that had a high likelihood of binding to 2 protective African class II alleles, namely DRB1*1301 which is protective in West Africa (Hill *et al.*, 1991), and DRB1*01 which is protective in East Africa (A. Hill, personal communication). Those with high predicted binding were synthesised for testing in East and West African donors.

2.14

PEPTIDE SYNTHESIS

2.14.1

Commercial Synthesis

Most peptides were manufactured commercially (Research Genetics, Huntsville, USA) according to the sequences provided. The peptide sequences used in the various studies are detailed in the relevant chapters. Peptide purity was checked using reverse phase High Pressure Liquid Chromatograhpy (HPLC), and the presence of a single dominant peak confirmed.

2.14.2

Synthesis in Oxford

Certain peptides were manufactured by myself and Mr. K. McIntyre in Oxford, using the automatic peptide synthesiser (Zinsser Analytic, Maidenhead, UK). 40mg of amino acids attached to resins (Zinsser Analytic, UK) were weighed into labelled reaction vessels, which were then transferred onto the reaction block of the synthesiser, and pre-wet with N-dimethylformamide (DMF) for about 10 minutes. Amino acids were reconstituted at a concentration of 0.5M in a solution of 0.45M 1-hydroxy-benxotriazole (HOBT) in DMF, except phenylalanine which was in HOBT in 1-methyl-1 pyrrolidinone (NMP). The amino acids were aliquoted into the amino acid containers on the synthesiser, and the amino acid sequences were programmed into the computer. Peptide synthesis occurred from carboxy- to amino-terminus, with the C terminal peptide attached to the resin. The programmed amino acid sequence was coupled to the C terminal peptide, one amino acid at a time until the sequence was complete. Peptides were then washed 4X with methanol, then 10X with dichloromethane (DCM), and left to dry completely under vacuum. 1ml of peptide cleavage solution was applied, and this solution was then transferred to a further 2mls of cleavage solution, and left overnight to cleave. The cleaved peptides were then mixed with ether, vortexed and centrifuged at 4°C, and pelleted. This procedure was repeated five times. The pellet was air dried after the final ether wash, dissolved in 5% acetic acid, transferred to glass vials, frozen on dry ice, and transferred onto a freeze drier and allowed to freeze dry completely. Peptides were then weighed and dissolved in DMSO, and later diluted to working concentrations. Peptide purity was checked using reverse phase High Pressure Liquid Chromatograhpy (HPLC), and the presence of a single dominant peak confirmed.

2.15

PROLIFERATION ASSAYS

The culture medium for all lymphoproliferation assays was α-MEM (Life Technologies, UK) with 10% batch tested NHS, supplemented with 2% penicillin/streptomycin and 1% L-glutamine (MEM10).

2.15.1

Proliferation Cell Preparation

PBMC were resuspended in MEM10 at $5x10^5$/ml and plated in triplicate to sextuplet in 96 well round bottomed tissue culture plates (Beckton Dickinson, France) at 200µl/well ($1x10^5$ cells) in the presence of peptides at 25µg/ml. PPD (5µg/ml) was used as a positive control, and medium alone was used as the background response. In certain cases, such as assessing proliferative responses of positively selected T cells, APCs were added to the wells to allow antigen presentation. They were added at an APC:responder cell ratio of 1:4.

2.15.2

Proliferation Transwell Experiments

0.4µm Cell Culture Inserts HD (Becton Dickinson, France) were placed in 24 well flat bottomed tissue culture plates. The cells were otherwise prepared in exactly the same way as above, and used at a concentration of $1x10^6$/ml in MEM10. Peptides were utilised at a final concentration of 25µg/ml. Culture plates were incubated for 5 days, and then plated onto 96 well round bottomed tissue culture plates in triplicate to sextuplet (as above). The effect of the cells present in the transwells was therefore assessed.

2.15.3

Proliferation Antagonism Assays

Two types of proliferation antagonism assays were performed using PBMC (Chapter 4). In the 'sequential pulsing' assay, half the PBMC were irradiated (2000 rads) and then incubated for 3 hours with the index peptide (25µg/ml). They were then washed 3X with RPMI, and incubated for 3 hours with the putative antagonist peptide (50µg/ml). They were then washed again, and mixed 1:1 with untreated PBMC as the responder cells at a final concentration of $2x10^6$ cells/well. In the 'separate pulsing' assay, the PBMC were prepulsed for 3 hours separately with either index peptide or putative antagonist (both at 50µg/ml), and added to untreated responder PBMC at a 1:1 ratio.

2.15.4

Proliferation Harvesting and Detection

Proliferation plates were incubated at 37°C/5% CO2 for between 5 and 10 days according to the time point being assessed. 1μCi [^{3}H]thymidine (Amersham Life Sciences, UK) was then added to each well of 96 well round bottomed plates which were incubated for a further 16 hours at 37°C/5% CO2. Plates were then automatically harvested onto spot-on cellulose 1205-401 filters (Wallac, Turku, Finland), and [^{3}H]thymidine incorporation was measured by liquid scintillation counting in a 1205 Betaplate ß-counter, for the Gambian studies. For the studies in Oxford the 96 well tissue culture plates were automatically harvested onto Select Unifilter^{TM}-96 GF/C plates (Packard, Pangbourne, UK) using the Packard FilterMate^{TM} harvester (Packard, UK). Plates were counted under 30μl/well Microscint-20^{TM} LSC-cocktail (Packard, UK) using the Microplate Scintillation and Luminescence Counter (Packard, UK). The plates were scored using TopCount^{TM} software.

2.15.5

Proliferation Scoring

The results were either expressed as mean counts per minute (CPM) ±standard error (SE), or as a stimulation index (SI) of counts per minute according to the following formula: mean test CPM / mean background CPM. An SI value of >2 was taken as the cut off value for a positive response in the study in The Gambia (Chapter 3) (Bennett and Riley, 1992).

2.16

STATISTICAL METHODS

The statistical table for scoring positive ELISPOT wells was compiled by Drs. W. Reece and P. Milligan in Oxford. The analysis for protection was performed by Amanda Ross, the statistician in Kilifi, Kenya. Drs. M. Gravenor and A. Cervino in Oxford helped with the analysis of other aspects of the data.

2.16.1

Statistical Scoring Method for ELISPOT Assays

Statistical analysis for comparing background (unstimulated) to antigen stimulated wells were compiled into a statistical significance table (Table 2.1) by Drs. P Milligan and W. Reece according to a number of mathematical assumptions (Sokal and Rohlf, 1995). It assumes that if the control unstimulated cells have C spots, and the peptide specific cells have T spots, then each test well in the plate can have C + T spots. Assuming that the probability of each spot (C + T) appearing in two different wells is the same, the statistical table assesses the likelihood of T or more spots appearing in the test well. If the frequency of likelihood is less than 5%, then the probability of a spot appearing in the test well must be greater than that of a spot appearing in the control well within 95% confidence. Where test wells are scored positive in this thesis, they have fallen within this 95% confidence interval, and thus have a $\leq$5% probability that they arose by chance.

2.16.2

T Cell Assay Correlation Analysis, The Gambia (Chapter 3)

Statistical analysis comparing responses to the 3 T cell assays (*ex-vivo* ELISPOT, cultured ELISPOT and proliferation) was performed by Dr. M. Gravenor in Oxford. A simulation approach was used to test whether the pattern of response to each peptide was correlated over the three assays. For each donor he summarised the correlation between a pair of assays by counting the number of times a positive result was found for the corresponding peptides. The average value was calculated over all donors for each pair of assay comparisons. The distribution of this average index under the null hypothesis of no correlation was calculated using binomial distributions with n = 48 donors, and the probability of a success estimated from the average number of positive responses in each assay. χ^2 tests were used to assess associations between antibody level, categorised as low (< 4μg/ml), medium (4-10μg/ml) and high (> 10μg/ml), blood smears (positive or negative), and assay responses (responds to at least one peptide or no response). In addition, proliferative stimulation index values were compared directly with Ab levels (μg/ml) to assess for an association.

2.16.3

Effects of Depleting Cells / Antagonism Assays (Chapters 3 & 4)

The effect of depleting cell populations expressing various activation markers from PBMC (Chapter 3) were assessed using student's t-tests where enough donors were tested. The proliferation counts were log transformed for this analysis. Student's t-tests were also used to assess the effect of adding putative antagonistic peptides to assays (Chapter 4).

2.16.4

East / West Africa & Adults / Children Comparisons (Chapters 5 & 6)

The comparisons of responses between East and West Africans and adults and children was performed by myself, with the help of Dr. A. Cervino in Oxford. Proportions of donors responding in different groups were compared used standard chi squared analysis, having first established that the background levels were comparable. Where the numbers in each group for comparison were very small, Fisher's p-value value for 2-tailed analysis was used. The precursor frequency values (SFU / 10^6 PBMC) were compared using t-tests to look for evidence of a significant difference between groups.

2.16.5

Analysis for Protection (Chapter 6)

Statistical analysis for protection was performed by Amanda Ross, the statistician in Kilifi, since the data is stored on a data base in Kilifi and is confidential at present. Analysis was performed using the STATA version 6 (STATA Corporation, TX, USA) computer programme. The endpoints for the analysis were chosen before the analysis began for the following 3 parameters:

Number of donors with parasitaemia over 2 months (any parasitaemia and $>10^4/\mu l$)

Number of donors with parasitaemia over 6 months (any parasitaemia and $>10^4/\mu l$)

A delay in time to first parasitaemia (any parasitaemia and $>10^4/\mu l$)

Fisher's exact tests were used to compare the proportions of donors with at least one episode of parasitaemia who had specific TRAP epitope T cell responses with those who did not have the response (Results 6.3.4 and 6.3.5). The mean total SFU values were compared for those who subsequently did and did not have an episode using two sample t-tests (see Results 6.3.6). Kaplan-Meier estimates of the proportion of donors remaining episode free were plotted against time following the cross-sectional analysis of TRAP ELISPOT responses. Log-rank tests were used to compare survival experience for those donors with particular TRAP T cell epitope responses to those without (Results 6.3.4 and 6.3.5). Maentel-Haenzel tests for homogeneity were used to assess whether there was evidence of a significantly different effect of having a TRAP response in different age groups (Results 6.3.5).

The relationship between total SFU and time to first parasitaemia appeared to be non linear, thus total SFU values were divided into 4 categories (< 0, 0-49, 50-99, ≥ 100) to look for Kaplan-Meier survival, rather than assessed as a continuous variable (Results 6.3.6). Cox's proportional hazards were used for analysis of total SFU and time to first episode of parasitaemia. The estimated effect of each total SFU category was presented as an estimated hazard ratio for an individual in each category compared to the baseline group (those with total SFU <0). It was assumed for this analysis that the effect of total SFU remained constant with time. A hazard ratio <1 meant that the hazard was less than the baseline category, and >1 meant greater than the baseline group. Differences in time to first episode between the categories were investigated using a likelihood ratio test, both univariately and after adjusting for age.

Figure 2.1

						95%												99.9%										
0	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
2	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
3	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
4	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
5	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
6	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
7	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
8	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
9	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
10	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
11	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
12	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
13	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
14	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
15	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
16	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
17	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
18	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46
19	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
20	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
21	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
22	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
23	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
24	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52
25	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
26	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
27	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
28	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
29	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
30	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58
31	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
32	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
33	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61
34	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
35	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63
36	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
37	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
38	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
39	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67
40	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
41	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
42	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
43	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
44	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
45	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73
46	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
47	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
48	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76
49	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77
50	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78
51	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79
52	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
53	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81
54	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82
55	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
56	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84

Section of statistical table used to score ELISPOT plates for positive responses. The number of spots (SFU) in the test wells (along the top) are compared to the number in the background wells (down the side) and a test response is only considered positive if it falls in the 95% probability and above (shaded boxes).

CHAPTER 3

DIFFERENT T CELL EFFECTOR FUNCTIONS TO CS PROTEIN EPITOPES IN ADULT GAMBIANS: THE ROLE OF DISTINCT MEMORY T CELL SUBSETS

3.1

INTRODUCTION

The circumsporozoite protein is the most widely studied of all the pre-erythrocytic antigens, and natural immunity to CS protein has been comprehensively assessed in field studies in malaria endemic regions of the world (reviewed in Chapter 1). There is extensive data supporting a protective role in animals, but more limited evidence for humans. Most human field studies of CD4 T cell mediated immunity have focused on lymphoproliferative responses to peptides and antigens (Good *et al.*, 1988d, Hoffman *et al.*, 1989b, de Groot *et al.*, 1989, Zevering *et al.*, 1990), yet none have convincingly shown an association between proliferative responses to CS protein and protection against disease. Responses in such studies are generally low grade, and widespread nonresponsiveness has been a consistent observation.

Few studies have measured for IFN-γ release by T cells, even though IFN-γ may be the cytokine by which T cells mediate their protective effect (reviewed in Chapter 1). In those studies which have assessed IFN-γ release in response to CS protein, it has been done by ELISA, and no association between lymphoproliferation and IFN-γ release is seen (Riley *et al.*, 1990). This lack of association between proliferation and IFN-γ release is also a feature of responses to the blood stage antigen MSP-1 (Riley *et al.*, 1992, Riley *et al.*, 1993), and is seen in other infectious diseases such as *Mycobacterium leprae* (Mutis *et al.*, 1993). This raises the questions of what role such proliferative responses play in protective immunity, and whether the IFN-γ responses are a separate entity. Indeed, recent studies have demonstrated that memory T cells may selectively perform different effector functions such as lymphokine secretion (Mosmann and Coffman, 1989, Street and Mossman, 1991,

Hollsberg *et al.*, 1995, Sallusto *et al.*, 1999), or cytotoxicity (Hollsberg *et al.*, 1995), and may also differ in their susceptibility to apoptotic signals (Varadhachary *et al.*, 1997, Zhang *et al.*, 1997), and in rapidity of response (Swain, 1991). Moreover, a recent study in Gabonese children showed a protective association between cultured (day 6) IFN-γ responses to liver stage antigen 1 (LSA-1) peptides and recurrence of infection, whilst proliferative responses to the same peptides were not protective (Luty *et al.*, 1999).

The enzyme linked immunospot (ELISPOT) assay has been refined to allow the identification of T cells which can secrete IFN-γ within 6 hours of antigen stimulation (Lalvani *et al.*, 1997). The rapidity of this response makes it a prime candidate for a protective role during the short time period (average 5-10 days) during which the parasites are found inside liver cells. Indeed, rapid IFN-γ ELISPOT responses to CS protein have been correlated with protection in the mouse model of malaria (Plebanski *et al.*, 1998). Additional information can be gained from the highly sensitive ELISPOT since it determines the precise number of IFN-γ producing cells circulating in peripheral blood. The ELISPOT assay is also less time consuming (overnight versus 5 days) than the proliferation assay, and does not require the use of radioactivity which is not always available in the tropics. It is a robust, simple and sensitive technique, and can be used to screen for multiple epitope responses at one time, and is thus a convenient assay to employ in large scale field studies of malaria and other infectious diseases.

3.2
STUDY DESIGN
3.2.1
Rationale

This study was designed to address whether such rapid T cell effector function, as measured by IFN-γ ELISPOT assay, focuses on the same T cell epitopes as those identified by proliferative assays. I performed three T cell assays simultaneously in malaria exposed Gambian adults to assess immunity to a series of 15mer CS protein derived epitopes. Thus, lymphoproliferation, rapid IFN-γ ELISPOT using freshly isolated PBMC

(*ex-vivo* ELISPOT), and IFN-γ ELISPOT assays after re-stimulation and expansion *in vitro* (cultured ELISPOT) were performed in response to the 8 CS protein epitopes selected for the study (Figure 3.1). It is not known if anti-CS Ab levels correlate with IFN-γ T cell responses, and there are conflicting reports as to whether Ab responses correlate with proliferation in natural immunity to CS protein (Good *et al.*, 1988d, Hoffman *et al.*, 1989b, de Groot *et al.*, 1989, Riley *et al.*, 1990). Thus, serum Ab levels to recombinant CS protein were assessed in parallel for each donor by ELISA, to look for a correlation between the 3 T cell assays. Malaria blood films were also performed on all donors to see if there was any relationship between blood film positivity and T cell responses to CS.

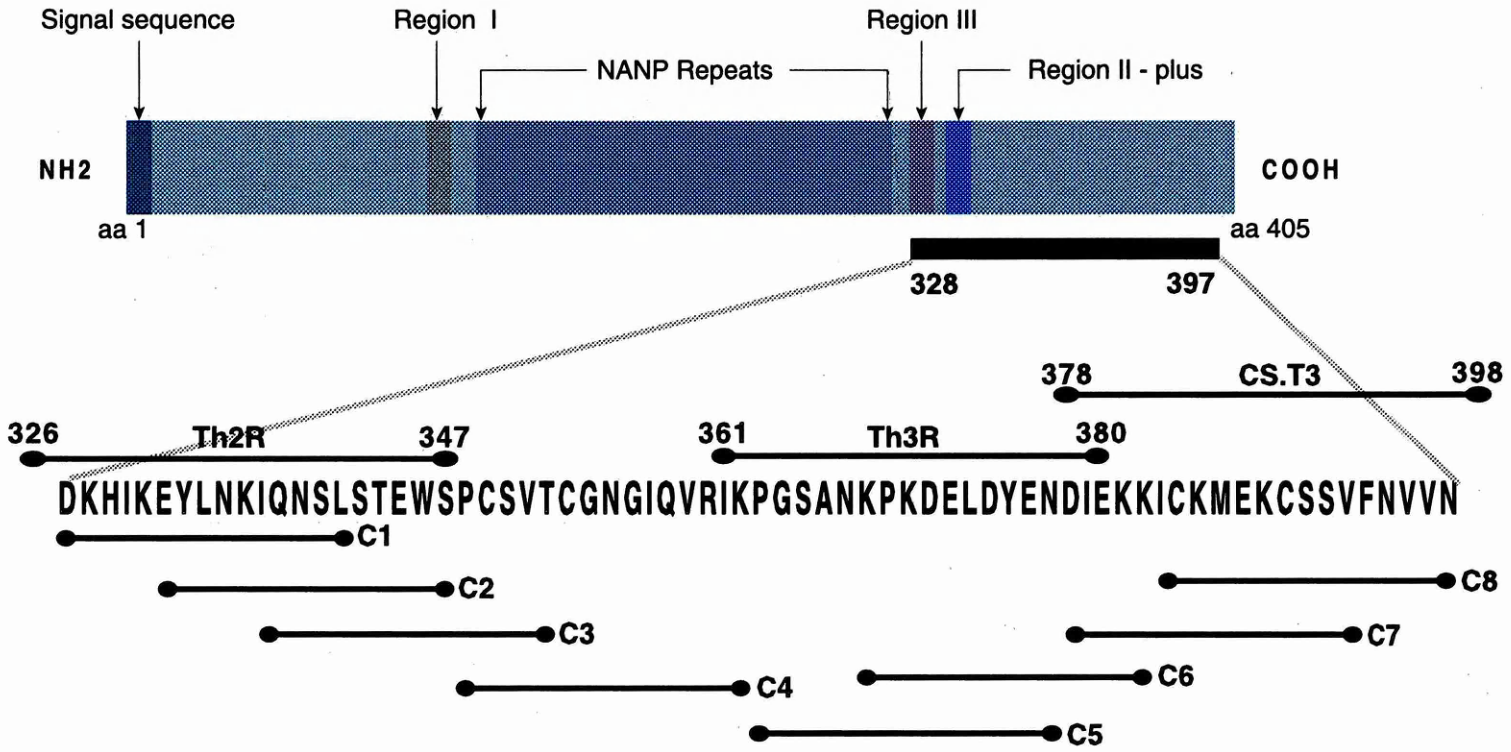
3.2.2

Study Site and Volunteers

Peripheral blood mononuclear cells (PBMCs) were collected from 50 healthy adult male volunteers (donors D1-D50) from Dampha Kunda, Upper River Division, The Gambia between February and March 1997 (Figure 3.2). This represents the middle of the dry season when malaria transmission rates are low. The malaria season begins 5 months later after the rains commence, with high malaria transmission rates occurring between August and November. Forty eight donor samples were used for the main comparative study of 3 T cell assays (donors D1-D48), and the remaining 2 donors (D49 and D50) were tested in limited assays only (see below).

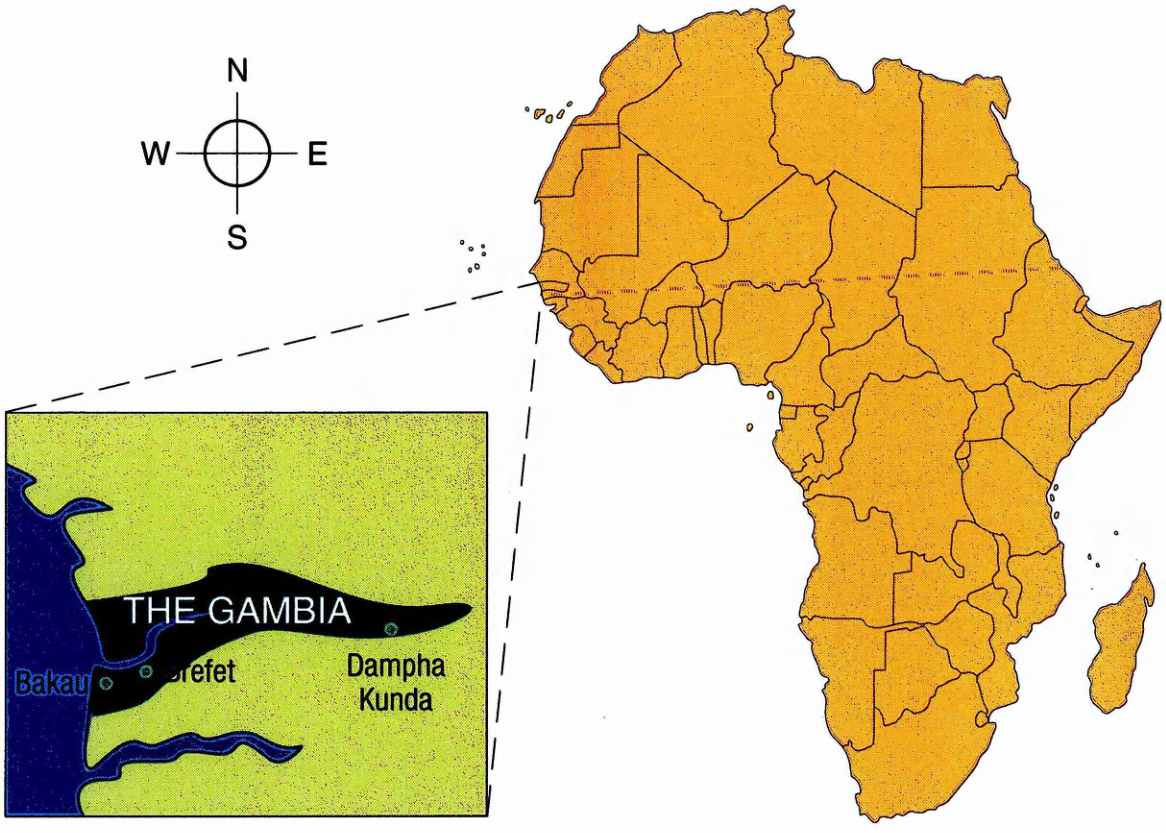
Twenty malaria naïve adult donors were recruited from the laboratory in Oxford for the assessment of responses of naïve donors to peptides from CS protein. A detailed travel history was taken from each of these donors to confirm that they had never been exposed to malaria. A further 15 healthy adult donors were recruited from the staff at the John Radcliffe Hospital regardless of previous malaria exposure to test the effects of CD38 / CCR7 depletion to common recall antigens.

Figure 3.1



Eight peptides (C1-C8) spanning the carboxy-terminus of CS were selected for the study. The immunodominant CD4 T cell epitope regions of CS, denoted Th2R, Th3R and CS.T3, are also indicated on the diagram.

Figure 3.2



Healthy adult donors were recruited from 3 sites in The Gambia: Bakau, Brefet and Dampha Kunda. The 3 Gambian studies (Chapters 3, 4, and 5) used donors from different study sites, and the details are given in the relevant sections of the text.

3.2.3

Peptides

Thirty seven 15mer peptides (overlapping by 5 residues) extending from the NANP repeat region to the carboxy terminus of CS protein (aa 273-412) from strains NF54 and 7G8 were synthesised commercially. All peptides were tested in IFN-γ ELISPOT in five randomly selected Gambian adults (Table 3.1). Eight peptides spanning residues 328-397 (C1-C8) were selected for the study (Figure 3.1), consisting of a combination of NF54 (peptides C1-C4, C7-C8) and 7G8 strain (C4-C8) derived sequences. These two strains vary by 6 nucleotide substitutions across residues 328 -397, all of which lead to amino acid changes (Caspers *et al.*, 1989). Peptides C4, C7 and C8 are identical for NF54 and 7G8. The relative frequencies of NF54 and 7G8 variants in the Basse region of The Gambia in November 1997 were as follows: 7G8 Th2R epitope 0.12, NF54 Th2R epitope 0.07, 7G8 Th3R epitope 0.05, NF54 Th3R epitope 0.26 (A. Alloueche, personal communication). Peptides corresponding to the known Th2R (aa 326-347, called peptide V6) and Th3R (aa 361-380) epitope regions of CS protein clone NF54 were similarly synthesized commercially (Figure 3.1). All peptides were reconstituted in RPMI-1640 (Gibco, Glasgow, UK), and RPMI was used as the negative control in all assays. Four peptide pools, consisting of two non-overlapping peptides per pool, were prepared in which to culture cells for a later IFN-γ ELISPOT assay: pool A contained peptides C1 and C4; pool B peptides C2 and C6; pool C peptides C3 and C7; and pool D peptides C5 and C8 (see Figure 3.1 for location of peptides). Peptide pools were added to the cells for culture to give a concentration of 25μg/ml per peptide. Purified protein derivative (PPD) (Statens Seruminstitut, Copenhagen, Denmark) was used as a generally positive control.

3.2.4

Statistical Methods

Statistical analysis for this study was done by Dr M Gravenor. A simulation approach was used to test whether the patterns of responses to each peptide were correlated over the *ex-vivo* ELISPOT, cultured ELISPOT and proliferative assays. For each donor he summarised the correlation between a pair of assays by counting the number of times the

Table 3.1

Peptide	1st aa	NF54 or 7G8	Donor T1	T2	T3	T4	T5	
1	273	Both						
2	278	Both					■	
3	283	Both						
4	288	Both						
5	293	Both						
6	298	Both						
7	303	7G8						
27		NF54						
8	308	7G8						
28		NF54						
9	313	7G8						
29		NF54						
10	318	7G8						
33		NF54						
11	323	7G8						
34		NF54	■					
12	328	7G8	■					
35		NF54		■				* C 1
13	333	7G8	■					
36		NF54	■					* C 2
14	338	7G8						
37		NF54	■					* C 3
15	343	Both						
16	348	Both			■			* C 4
17	453	Both					■	
18	358	Both						
19	363	7G8		■				* C 5
38		NF54						
20	368	7G8	■		■			* C 6
39		NF54						
21	373	7G8						
40		NF54						
22	378	Both						* C 7
23	383	Both				■		* C 8
24	388	Both						
25	393	Both						
26	398	Both	■				■	
PPD	NA	NA	■	■	■	■	■	

IFN-γ ELISPOT pilot study testing 37 15mer CS peptides from strains NF54 and 7G8 of circumsporozoite protein in 5 Gambian donors. The peptides marked with an asterisk (C1-C8) were selected for the main study.

same result was found for the corresponding peptides. This is a maximum of 8 when the individual shows the same response to each peptide in each assay. The average value was calculated over all donors for each pair of assay comparisons. The distribution of this average index under the null hypothesis of no correlation was calculated using binomial distributions with n = 48, and the probability of a success estimated from the average number of positive responses in each assay.

Standard chi square tests were used to assess associations between antibody level, categorised as low (<4μg/ml), medium (4-10μg/ml) and high (>10μg/ml), blood smears (positive or negative), and assay responses (responds to at least one peptide or no response). In addition, proliferative stimulation index values were compared directly with Ab levels (μg/ml) to assess for an association. The effects of depleting cells expressing various surface markers were assessed using paired students' t-tests. The proliferation counts were log transformed for this comparison.

3.3

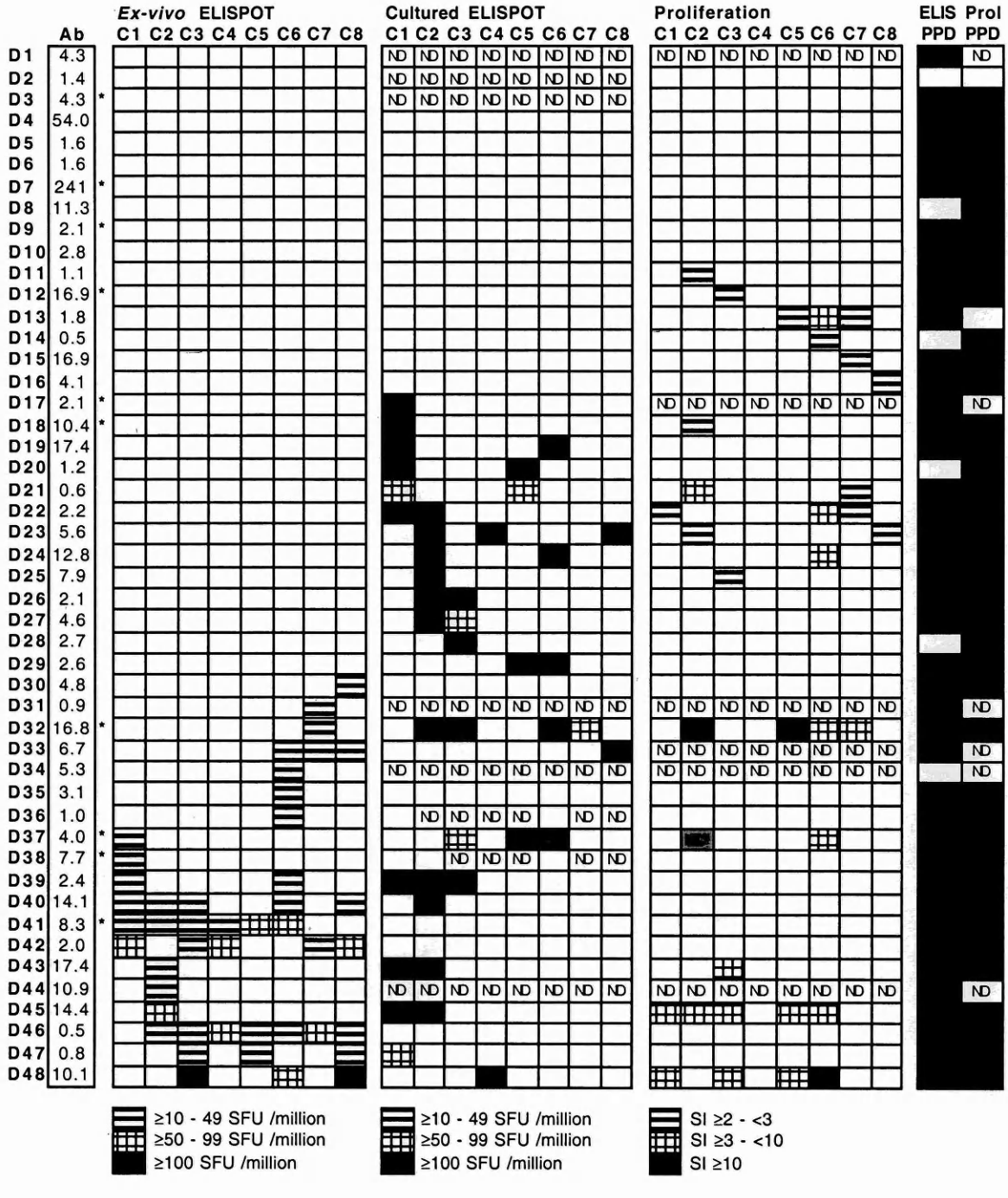
RESULTS

3.3.1

Lack of Correlation Between Responses to CS Protein Determined by Simultaneous Assessment of Three Different T Cell Effector Functions

48 donors were tested for responses to a panel of 8 overlapping peptides C1 to C8 spanning residues 328-397 of the carboxy-terminus of CS protein (Figure 3.1) in *ex-vivo* ELISPOT, cultured ELISPOT, and lymphoproliferative assays. PPD was used as a positive control antigen being frequently positive in this population. Eighty four percent (32/38) of the 38 donors for whom there were sufficient cells to perform all 3 assays responded to one or more CS peptide, and all but two donors gave a positive response to PPD in *ex-vivo* ELISPOT or proliferation (Table 3.2). Twenty malaria naïve adult donors recruited in Oxford were tested for *ex-vivo* IFN-γ ELISPOT responses to peptides C1 to C8, and no positive responses were found, whilst all naïve donors responded to PPD (not shown).

Table 3.2



*Summary of ex-vivo ELISPOT, cultured ELISPOT, proliferative responses, and anti-CS antibody levels (anti-R32LR) for 48 Gambian donors (D1 to D48). Filled in squares represent a positive response, and are shaded according to the magnitude of the response (see key). Antibody levels are expressed in μg/ml; ND = not determined, * = malaria blood film positive.*

Five malaria naïve donors were tested in cultured ELISPOT, and four donors in lymphoproliferative assays, and again no positive responses were observed to the 8 CS peptides (not shown).

More than half of the donors who gave a positive response did so to more than one peptide. The maximum number of peptides to which an individual responded was 7 in the *ex-vivo* ELISPOT assay (donor D46), 4 in the cultured ELISPOT assay (donor D32), and 5 by lymphoproliferation (donor D45) (Table 3.2). For each T cell effector function those donors that gave multiple responses were different (Table 3.2). It was possible that a response to two overlapping peptides in a particular donor represented a single response to a shared T cell site, for example D42 responded in the *ex-vivo* ELISPOT to the overlapping peptides C7 (aa 378-392) and C8 (aa 383-397). However, responses were also observed to each peptide individually over the 3 assays, for example D31 and D32 responded to C7 but not C8; D30 and D40 responded to C8 but not C7, suggesting that each peptide must also represent a T cell epitope in its own right.

I found that 40% of donors (19/48) responded to one or more peptide in the *ex-vivo* ELISPOT assay, 55% (22/40) in the cultured ELISPOT assay, and 40% (17/42) by proliferation. Eleven (23%) of the donors tested in *ex-vivo* ELISPOT responded to 1 peptide only, and 8 donors (17%) responded to more than 1 peptide. Eight (20%) of the donors tested by cultured ELISPOT responded to one peptide only, and 14 (35%) to more than 1 peptide. Nine donors (21%) proliferated to one peptide and 8 (19%) donors gave multiple responses by lymphoproliferation. Thus cultured ELISPOT gave double the number of multiple epitope responders (35%) compared to the other 2 assays (17% & 19%). The cultured ELISPOT responses were of a much higher magnitude (mean 224 ±30.6 SE) than the *ex-vivo* responses (mean 39 ±5.3 SE) (Table 3.3), probably reflecting their 2 week expansion period *in vitro* and differences in the population measured (see later). The lymphoproliferative responses were generally low (mean SI 4.6 ±0.7 SE).

Table 3.3

		Ex-vivo ELISPOT									Cultured ELISPOT								Proliferation								
	Ab	C1	C2	C3	C4	C5	C6	C7	C8	PPD	C1	C2	C3	C4	C5	C6	C7	C8	C1	C2	C3	C4	C5	C6	C7	C8	PPD
D29	2.6									235					150	170											9.2
D30	4.8								13	160																	96
D32	16.8							23		155		1000	130			820	90			15			22	5	5		200
D35	3.1						30			355																	18
D37	4.0	15								315			90		130	190				2				4			39
D39	2.4	25					23			235	170	150	200														41
D40	14.1	25	43	20			25		22	90		100															117
D41	8.3	23	13	15	15	48	40			135																	2.8
D42	2.0	88		43	80			43	50	50																	133
D43	17.4		25							30	350	170									3						41
D45	14.4		33							130	380	360							4	6	4		3	3			44
D46	0.5		13	30	55	15	20	58	13	50																	10
D47	0.8			6		9			7	75	50																35
D48	10.1			41			36		55	258				460					4		4		5	15			95

Examples of ex-vivo ELISPOT (SFU / million PBMC), cultured ELISPOT (SFU / million PBMC) and proliferation (stimulation index) values for 10 of the Gambian donors. The cultured ELISPOT responses were generally an order of magnitude greater than ex-vivo responses, and the lymphoproliferative responses were generally low to individual epitopes.

Low grade T cell responses are characteristic of malaria induced natural immunity both to pre-erythrocytic stage (Good *et al.*, 1988d, Riley *et al.*, 1990) and blood stage antigens (Riley *et al.*, 1992, Lee *et al.*, 2000), a phenomenon which is discussed in detail in the introduction (Chapter 1).

A positive response to each peptide in the *ex-vivo* ELISPOT was not significantly correlated with the corresponding peptide response in proliferation ($p = 0.57$). This result is in agreement with the lack of correlation between proliferative responses and IFN-γ release seen in response to the 2 blood stage malaria antigens Pf155/ring-infected erythrocyte surface antigen (RESA) (Troye-Blomberg *et al.*, 1990) and merozoite surface protein 1 (*Pf*MSP1) (Riley *et al.*, 1993) in naturally exposed Gambian adults. I also failed to find a correlation between the *ex-vivo* and cultured ELISPOT responses ($p = 0.65$). One might predict that proliferation and cultured ELISPOT would detect the same T cells, since both assays measure cells re-stimulated in culture. However, in this study the pattern of peptide responses was not significantly correlated over the two assays ($p = 0.12$). Thus, for no individual peptide did a positive response for one of the three T cell effector functions correlate significantly with a positive response to another.

The PBMC were stimulated differently for each of the 3 T cell assays, which may contribute to their lack of correlation, for example by preferentially promoting the activation of a different T cell subset. Thus, the cultured ELISPOT cells were cultured for 14 days in MEM-10 medium in a 48 well tissue culture plate and IL-2 was added on 2 occasions. The proliferating cells were also cultured in MEM-10, but only for 5 days, and no IL-2 was used. The ELISPOT assays using the *ex-vivo* cells and cultured cells were performed in exactly the same way except that less cultured cells (1×10^5) were added per well in the assay since after culture the reactive cells would have otherwise been too numerous to score visually.

3.3.2

Different Immunodominant Epitopes are the Target of Diverse T Cell Effector Functions

Lymphoproliferative assays in naturally exposed donors have identified 3 immunodominant T cell epitope domains located outside the repetitive region of CS, called the Th2R (aa 326-347), Th3R (aa 361-380) (Good *et al.*, 1988d, Shi *et al.*, 1992), and CS.T3 (aa 378-398) regions (Sinigaglia *et al.*, 1988a). All of these domains are covered by the peptides assayed in this study (Figure 3.1). Up to 25% of naturally exposed adults respond to the Th2R region and 43% to the Th3R region of CS protein (Good *et al.*, 1988d). Sinigaglia and colleagues found that 56% of naturally exposed adults from the Ivory Coast respond to region CS.T3, and a surprisingly high proportion (one third) of malaria naïves also had a proliferative response to CS.T3 in this study (Sinigaglia *et al.*, 1988a). However, Good and colleagues found no proliferative responses to the equivalent peptide 27 (aa 380-399) in 35 Gambian adult volunteers (Good *et al.*, 1988d). Similarly, Hoffman and colleagues found no proliferative responses to CS protein residues 381-400, and only 1 responder to residues 376-395, in a study of 28 Kenyan adults (Hoffman *et al.*, 1989b).

The most frequent positive response in *ex-vivo* ELISPOT in this study was to CS peptide C6 (aa 368-382) (19% donors) which overlaps the Th3R region (aa 361-380); whereas in cultured ELISPOT it was to peptide C2 (aa 333-347) (27% donors) which is contained within the Th2R region (aa 326-347). In assays of T cell proliferation, peptide C6 (Th2R) (19% of donors) was recognised most frequently, with C2 (Th2R) next in frequency (17% of donors). Peptide C8 (aa 383-397) was the second most commonly recognised epitope in *ex-vivo* ELISPOT (15% of donors) in our study. This peptide corresponds to the CS.T3 (aa 378-398) region which is recognised in association with at least 7 different class II HLA DR types (Sinigaglia *et al.*, 1988b), although the minimal epitope corresponding to C8 is recognised by only 3 of these 7 DR types. Thus, each assay gives a different hierarchy of epitope responses, although individual epitopes are recognised at frequencies similar to previous studies.

CD4 and CD8 depletion studies confirmed that IFN-γ ELISPOT responses to epitopes C1, C2, C3 and C6 were CD4 mediated (Figures 3.3 A-D). It is probable that the other 4 epitopes are also CD4 epitopes in nature. Similar depletion studies for cultured ELISPOT responses to 15mer epitopes spanning the entire carboxy-terminus of CS protein using PBMC from RTS,S vaccinated Europeans confirmed that the responses were CD4 mediated in 37/40 cases tested (W. Reece, personal communication).

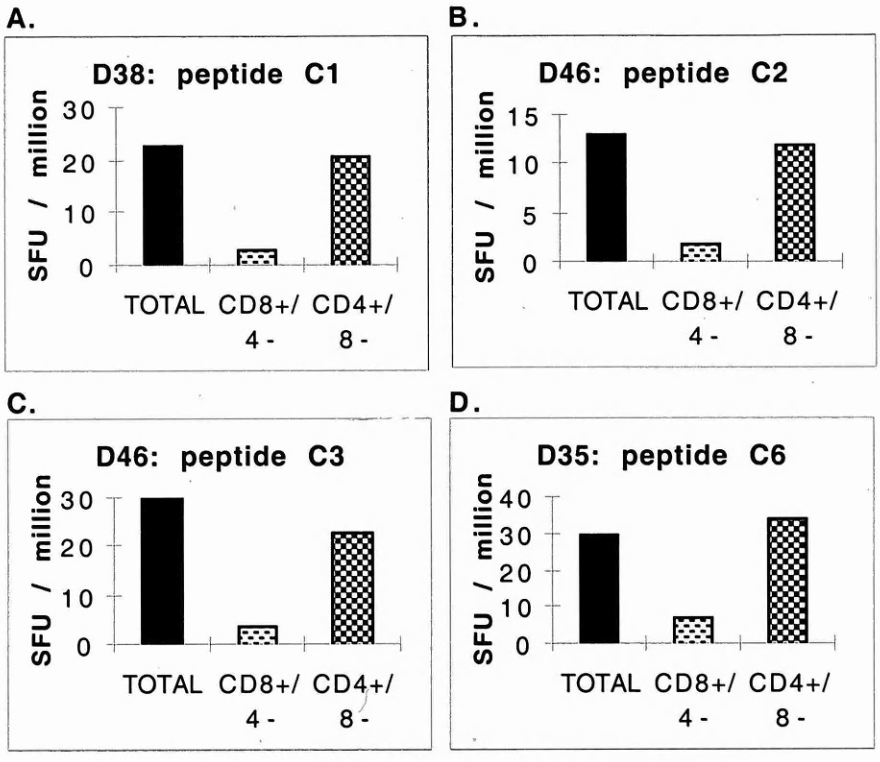
3.3.3

Correlates with Antibody Responses to CS Protein in Naturally Exposed Adults

Several studies of natural immunity to CS protein find no correlation between proliferation and anti-CS Ab levels (Riley *et al.*, 1990, Good *et al.*, 1988d, de Groot *et al.*, 1989). However, in a separate study Hoffman and colleagues do observe a correlation (Hoffman *et al.*, 1989b). To explore this issue further, I tested for a correlation with anti-CS Ab levels and proliferation to the carboxy-terminus of CS. Since I also assessed for new T cell effector functions (*ex-vivo* and cultured IFN-γ secretion), it was important to determine whether a correlation with Ab levels might now be found. Anti-CS antibody levels were tested for the 48 donors above, and ranged from 0.5 to 241μg/ml (mean 11.5 ±4.9 SE, Table 3.2). Forty six percent of donors had low anti-CS Ab levels of <4μg/ml which are generally considered to be background levels, 25% had moderate levels between 4 and 10μg/ml, and 29% had high levels of >10μg/ml.

A comparison of Ab levels with positive responses in the 3 assays demonstrated that there was no correlation between Ab levels and *ex-vivo* or cultured ELISPOT (χ^2 test, $p = 0.3$ and 0.4 respectively), but a borderline correlation was found for those who gave positive proliferative responses ($p = 0.05$). Thirty two percent of positive responders in the *ex-vivo* ELISPOT, 36% in cultured ELISPOT, and 47% of those whose T cells proliferated to the CS peptides had high Ab levels (>10μg/ml), with no significant correlation between high antibody production and a positive assay response in *ex-vivo* ELISPOT (χ^2 test $p = 0.8$) or cultured ELISPOT ($p = 0.6$).

Figure 3.3



CD4 and CD8 depletion studies showed that IFN-γ ELISPOT responses of undepleted PBMC (Total, black bars) to 4 peptides (C1, C2, C3 and C6) were lost in the CD4 depleted wells (stippled bars), but maintained in the CD8 depleted wells (chequered bars).

The correlation between Ab production and positive lymphoproliferation no longer reached significance ($p = 0.06$) when only those donors with high Ab levels were considered. Thus, although proliferation correlated rather weakly with Ab levels, no correlation with *ex-vivo* or cultured IFN-γ ELISPOT was found.

IFN-γ has been shown to be protective in animal models of malaria infection (Schofield *et al.*, 1987c, Maheshwari *et al.*, 1986), with evidence for a protective role in humans (Stoute *et al.*, 1997, Mellouk *et al.*, 1987). It was therefore considered to be an important cytokine to assess in our study of natural T cell immunity and correlates of antibody production. However, it was feasible that other cytokine responses, such as the Th2 type cytokine IL-4, might correlate with antibody levels. Indeed, Troye-Blomberg and colleagues find that

there is a correlation between IL-4 production and serum Ab levels to peptides derived from the blood stage antigen Pf155 / RESA in malaria (Troye-Blomberg *et al.*, 1990). Anti-CS Ab levels were thus assessed in parallel by IL-4 and IFN-γ ELISPOT to the immunodominant epitopes Th2R (aa 326-347) and Th3R (aa 361-380) of *Pf*NF54 strain of CS protein (see Figure 3.1) for 19 of the 48 donors recruited to this study. An additional two donors were also tested for IL-4 and IFN-γ responses to these 2 epitopes (D49 and D50) (Table 3.4). Two donors had a positive IL-4 ELISPOT response to the Th2R epitope, one of whom gave a positive IFN-γ response (Table 3.4). The three donors who produced IL-4 in response to the Th3R region also produced IFN-γ to the same epitope. The frequency of IFN-γ responders (29%) was greater than the IL-4 response rate (19%). No correlation was apparent between Ab levels and IL-4 ELISPOT responses in these donors, although the number of IL-4 responders was small (n = 4).

3.3.4

Parasitaemia Does Not Correlate with Immunological Parameters

All donors had thick and thin malaria blood films performed at the time of blood collection, and fourteen donors (29%) were found to be parasitaemic with asexual forms of *P. falciparum*. Four of the blood film positive donors had background anti-CS Ab levels (<4μg/ml), six had moderate levels (4-10μg/ml), and four donors had high antibody levels (>10μg/ml), with no correlation between smear positivity and Ab level (χ^2 test, p = 0.5). Similarly, there was no association between blood smear positivity and any of the three T cell assays employed in this study (*ex-vivo* ELISPOT p = 0.8, cultured ELISPOT p = 0.9, proliferation p = 0.7).

Table 3.4

		D1	D3	D4	D6	D7	D8	D10	D12	D15	D17	D19	D26	D28	D30	D31	D34	D40	D41	D47	D49	D50
Anti-CS Ab (μg / ml)		4.3	4.3	2.8	1.6	243	11	2.8	17	17	2.1	17	2.1	2.7	4.8	0.9	5.3	14	8.3	0.8	ND	ND
Th2R	IFN-γ											■			■						■	
PSDKHIKEYLNKIQNSLSTEWS	IL-4										▨										▨	
Th3R	IFN-γ				■							■		■	ND		ND				■	
IKPGSANKPKDELDYANDIE	IL-4											▨		▨							▨	

IFN- γ (black squares) and IL-4 (hatched squares) ex-vivo ELISPOT responses to the immunodominant Th2R and Th3R epitope regions of CS strain NF54 were assessed in parallel to anti-CS Ab levels for 21 donors. Filled in squares represent a positive response by ELISPOT, ND = not determined.

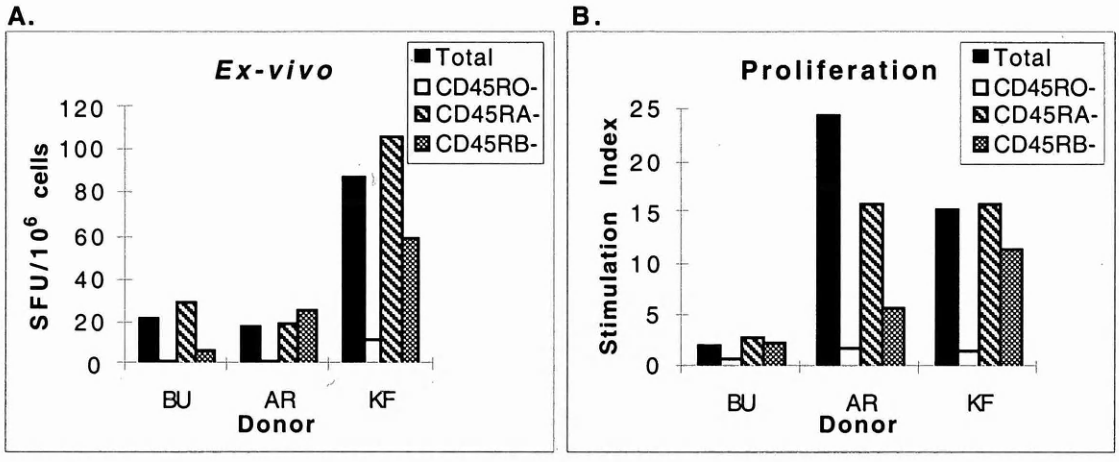
3.3.5

Effect of CD45RO, CD45RA and CD45RB Depletion of PBMC on the 3 T Cell Assays

The lack of correlation between epitope responses measured by these 3 T cell assays suggested that different memory T cell subtypes were involved. Differentiating memory T cells has however proved to be an elusive goal. Until recently, human isoforms of CD45 (a phosphatase involved in cell signalling) have provided the only means of identifying memory T cells. Thus, memory / effector T cells have been characterised as expressing a $CD45RA^{low}RO^{high}$ phenotype (Mackay, 1993), and naïve T cells express the converse $CD45RA^{high}RO^{low}$ phenotype. Indeed, memory T cells capable of proliferation or rapid IFN-γ secretion have been shown to be $CD45RO^{+}$ (Plebanski *et al.*, 1992, Lalvani *et al.*, 1997). I found that depletion of PBMC expressing CD45RA and CD45RO had the predicted effect on *ex-vivo* ELISPOT and proliferative responses to PPD. Thus, CD45RA depletion did not lead to a significant effect in ELISPOT ($n = 6$, $p = 0.49$) or proliferation assays ($n = 5$, $p = 0.11$) (Figure 3.4A and B, representative results for 3 donors). CD45RO depletion should cause a loss of memory T cells, and indeed depletion led to a significant decline in IFN-γ ELISPOT responses ($n = 7$, $p = 0.004$), although the decline observed in the proliferation assays was not significant ($n = 4$, $p = 0.08$), probably because only 4 donors were tested (Figure 3.4A and B, results for 3 donors).

Horgan and colleagues demonstrated that CD45RB expression may define different subsets of $CD45RO^{+}$ memory cells in humans which have differential abilities to proliferate and induce cytokines (Horgan *et al.*, 1994). Indeed, in rats CD45RB expression has been shown to identify a T cell subset within the $CD45RO^{+}$ group specifically capable of IFN-γ secretion (Swain, 1991). I was therefore interested to see whether the $CD45RB^{+}$ cells were responsible for rapid IFN-γ release detected by ELISPOT. As predicted, depleting CD45RB cells led to a significant decrease in the IFN-γ ELISPOT PPD response ($n = 8$, $p = 0.017$) (Figure 3.4B). The effect on lymphoproliferative responses was highly variable in the 7 donors tested ($p = 0.67$).

Figure 3.4



Effect of depleting cells expressing the surface markers CD45RO (white bars), CD45RA (hatched bars) and CD45RB (stippled bars) on the undepleted PBMC (Total, black bars) response to PPD by ex-vivo ELISPOT (A) and lymphoproliferation (B). Representative results for 3 donors.

3.3.6

CCR7$^+$ Cells are Required to Generate Cultured IFN-γ ELISPOT Responses, But Not *ex-vivo* IFN-γ ELISPOT or Proliferative Responses

Sallusto and colleagues used a mAb to demonstrate that human memory CD45RO$^+$ T cells can be divided into two functionally distinct subsets characterised by the presence or absence of expression of the chemokine receptor CCR7 (Sallusto *et al.*, 1999). The CCR7$^-$ group mediates rapid effector function, and is capable of rapid cytokine production including IFN-γ. I hypothesised that this T cell subgroup, denoted T_{EM} (effector memory), is a likely candidate mediator of *ex-vivo* ELISPOT responses. A second T cell group, denoted T_{CM} (central memory), is CCR7$^+$ and requires further *in-vivo* stimulation and culture prior to the production of cytokines such as IFN-γ. I predicted that CCR7$^+$ cells might be required to generate cultured ELISPOT responses.

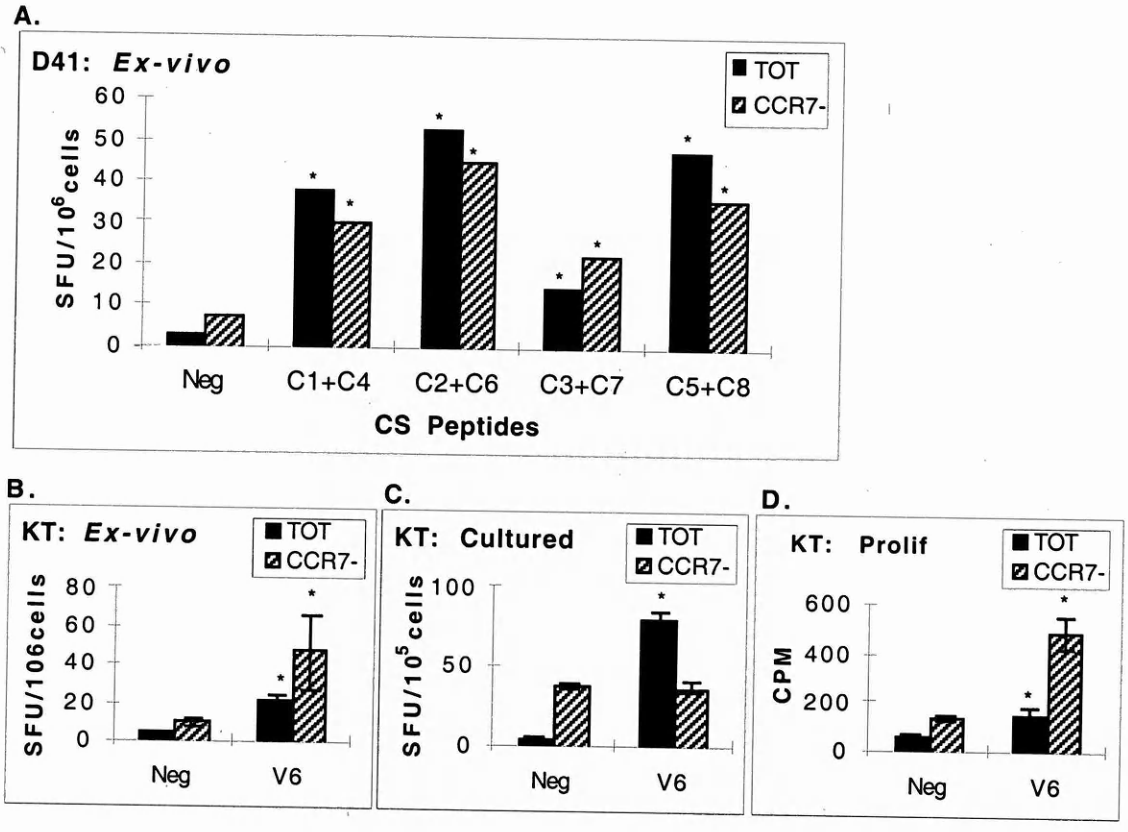
To test this hypothesis I obtained an aliquot of the CCR7 mAb used in the studies by Sallusto and colleagues. Frozen PBMC from donor D41 from The Gambian study were

defrosted and depleted for cells expressing CCR7 (see Methods). The cells were then tested in an overnight IFN-γ ELISPOT assay against 4 pools of the CS peptides C1 to C8. The undepleted PBMC responded to peptides C1-C6 in the original study, and the CCR7 depleted PBMC gave comparable ELISPOT responses to the equivalent peptide pools (Figure 3.5A). This confirmed that the *ex-vivo* ELISPOT required $CCR7^-$ cells, but not $CCR7^+$ cells to generate a response. Donor KT, who is known to respond to the CS derived Th2R malaria epitope V6, was depleted of PBMC expressing CCR7, and *ex-vivo* ELISPOT, cultured ELISPOT and proliferation assays were set up in parallel with undepleted and depleted cells. The *ex-vivo* ELISPOT response was comparable for undepleted and $CCR7^-$ PBMC (Figure 3.5B), whereas the cultured ELISPOT response which was positive for the undepleted PBMC, was significantly reduced following CCR7 depletion prior to culture (Figure 3.5C). The lymphoproliferative response was present in both undepleted and depleted cells with comparable stimulation indices of 2.4 and 3.4 respectively (Figure 3.5D).

This confirms the hypothesis that $CCR7^-$ cells mediate *ex-vivo* ELISPOT responses, and that cultured ELISPOT responses cannot be generated in the absence of $CCR7^+$ cells. Studies for 2 more donors to an HLA-A2 restricted influenza epitope and PPD showed the same trend with *ex-vivo* and proliferative responses present in the CCR7 depleted wells, but failure to generate cultured ELISPOT responses following $CCR7^+$ cell depletion (Figures 3.6 A-F). These different T cell subsets may therefore mediate different effector functions for MHC class I as well as class II restricted epitope responses.

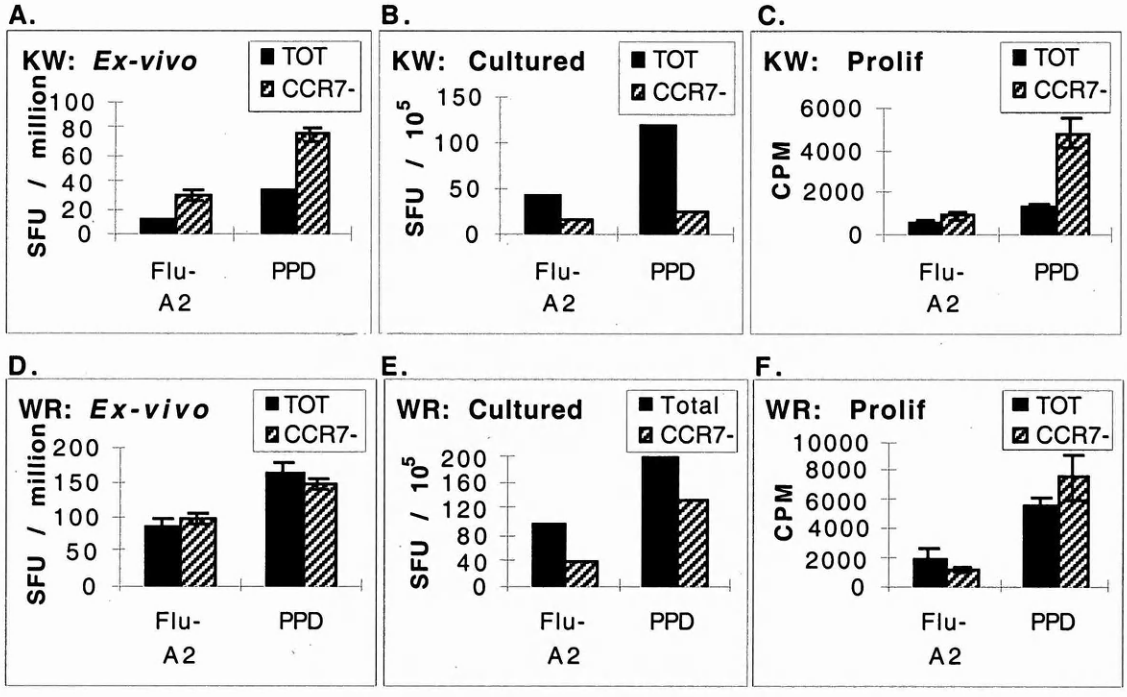
I thus have preliminary evidence in 4 donors supporting my hypothesis that the *ex-vivo* ELISPOT measures $CCR7^-$ circulating effector T cells (T_{EM} cells) in response to antigen, whilst the cultured ELISPOT detects a $CCR7^+$ central memory population (T_{CM} cells). The original study by Sallusto and colleagues showed that the $CCR7^+$ and $CCR7^-$ cells are capable of proliferation, and thus would not be expected to be decreased by CCR7 depletion if the same number of cells are used in the undepleted and depleted assays.

Figure 3.5



Effect of depletion of PBMC of cells expressing the chemokine receptor CCR7. IFN-γ ex-vivo ELISPOT responses to pools of CS protein peptides C1-C8 were maintained following depletion of donor D41 PBMC (A). CCR7 depletion of donor KT PBMC led to a significantly reduced cultured ELISPOT response (C, n = 2 experiments), with maintenance of ex-vivo ELISPOT (B, n = 2 experiments) and proliferative responses (D, n = 1 experiment) to a CS protein peptide V6 (Th2R epitope region variant). Undepleted PBMC (TOT) are indicated by black bars, and the CCR7 depleted (CCR7) response by hatched bars. Standard error bars are shown.

Figure 3.6



Effect of depleting PBMC expressing the chemokine receptor CCR7. PBMC from two donors (KW and WR) were depleted of cells expressing CCR7, and the effect of depletion on the 3 T cell assays to PPD and an HLA-A2 restricted CTL influenza epitope (Flu-A2) was assessed. Ex-vivo ELISPOT responses were maintained or enhanced (A and D), cultured ELISPOT responses decreased (B and E), and proliferative responses were unchanged or enhanced (C and F). Undepleted PBMC (TOT) are indicated by black bars, and the CCR7 depleted (CCR7) response by hatched bars. Standard error bars are shown.

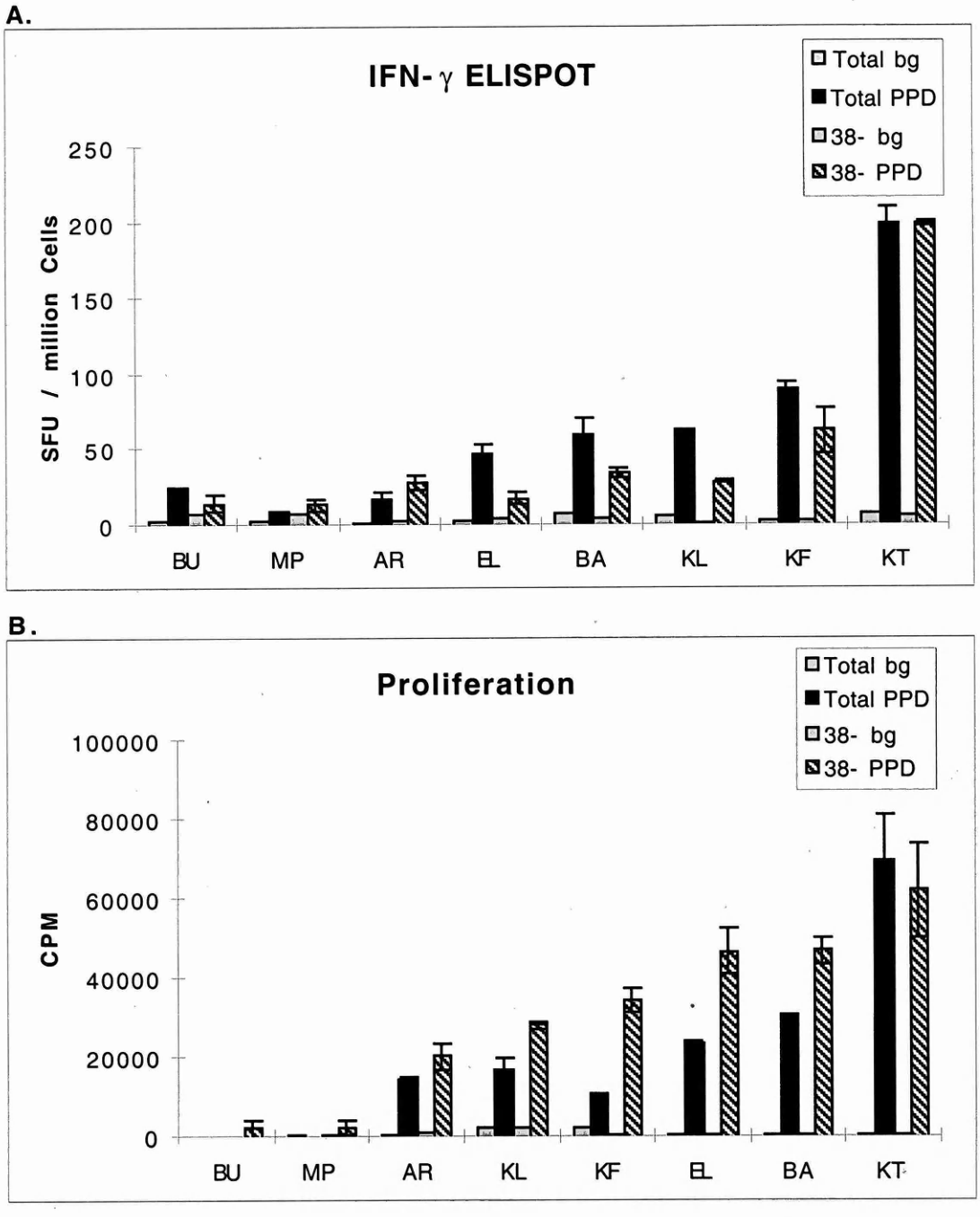
3.3.7

Proliferation and Rapid IFN-γ Responses to PPD are Differentially Regulated by $CD38^+$ Cells

CCR7 depletion can thus distinguish those cells mediating cultured ELISPOT responses, from *ex-vivo* ELISPOT and lymphoproliferative responses. However, the lack of correlation between IFN-γ production and proliferation still needed to be explained. In preliminary assays, PBMC depleted of those cells expressing the T cell activation markers CD27, NKA (EB6) and NKB (CD158b), failed to show consistent differences in reactivity in either proliferative or IFN-γ assays compared to undepleted PBMC (not shown). Expression of CD38 has been used as a marker of activation in human T cells (Funaro *et al.*, 1990), and CD38 ligation can induce early IFN-γ production in the absence of proliferation (Ausiello *et al.*, 1995). I thus hypothesised that depletion of cells expressing CD38 might differentiate *ex-vivo* IFN-γ ELISPOT and lymphoproliferative responses.

In order to explore the role of $CD38^+$ cells in the 3 different assays in more detail I analysed responses to the common recall antigen PPD. Surprisingly, I observed a reciprocal effect of CD38 depletion on *ex-vivo* IFN-γ ELISPOT compared to lymphoproliferation. In *ex-vivo* ELISPOT, depletion of CD38 cells led to a significant drop in PPD response in 5 out of 8 donors tested (Figure 3.7A) with no change in two donors, and an increase in one. The day 5 lymphoproliferative response to PPD was concomitantly increased in 7 out of 8 of these donors following CD38 depletion (Figure 3.7B). Cultured ELISPOT responses performed to PPD on two of these donors showed a drop in response on CD38 depletion in one donor, and no change in the other (not shown).

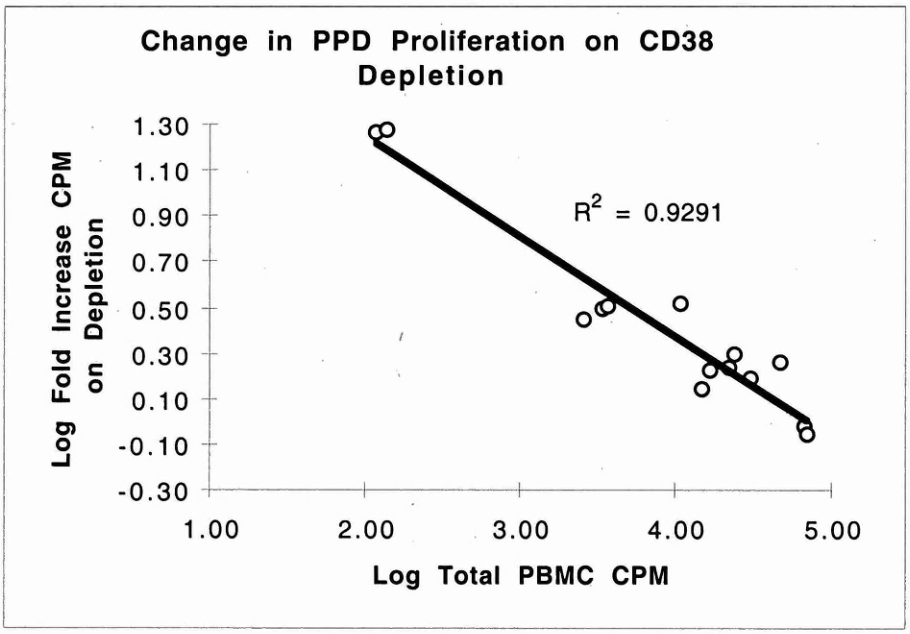
Figure 3.7



Effect of CD38 depletion of PBMC on the undepleted PBMC (black bars) response to PPD in 8 donors. Depletion of $CD38^+$ cells (hatched bars) led to decreased or unchanged ex-vivo ELISPOT responses (A), and significantly enhanced proliferative responses in most donors (B). Standard error bars are shown.

The effect of CD38 depletion was greatest when the initial PBMC proliferative response was low, and as the starting PBMC proliferative capacity increased, the effect of CD38 depletion became less pronounced. A plot of the log β-counts to PPD for the undepleted PBMC on the x-axis, against the log fold increase in count following CD38 depletion for 14 donors, gave a straight line ($R^2 = 0.93$, $p = 2x10^{-7}$) (Figure 3.8). This strongly suggested that the starting count was highly correlated to the subsequent increase in count following depletion.

Figure 3.8

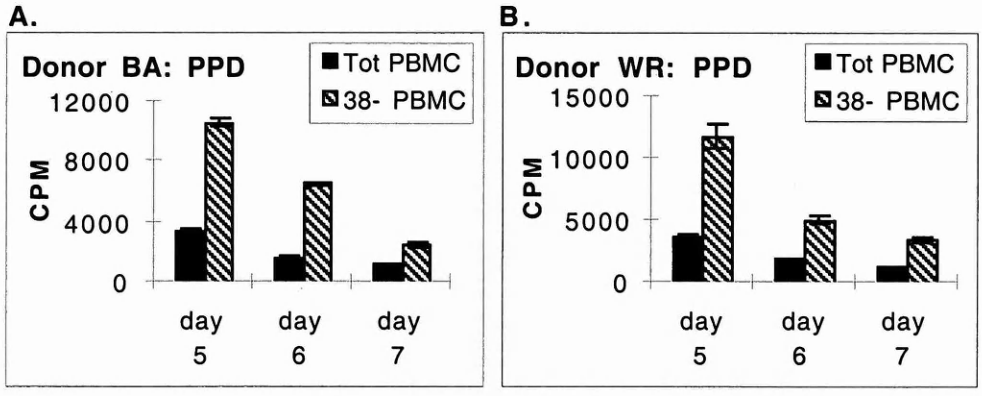


Effect of the initial PBMC proliferative value to PPD (Log Total PBMC CPM) on the subsequent increase in proliferation following CD38 depletion (Log fold increase CPM). A logarithmic plot for 14 donors gave a straight line, $R^2 = 0.93$, $p = 2x10^{-7}$. This suggested that the magnitude of the initial PBMC CPM was directly related to the effect CD38 depletion had. At the lowest starting CPM values depletion had the greatest effect, but if the starting values were high initially then depletion of CD38 cells had little or no effect.

Thus, where a donor gave poor proliferative responses to PPD (CPM <500), CD38 depletion led to marked enhancement of responses, suggesting that $CD38^+$ cells were actively suppressing the response. As the starting PBMC proliferative response increased (CPM $1x10^4$-$3x10^4$), the effect of CD38 depletion diminished. At the highest starting proliferative responses (CPM $7x10^4$), there was no effect following CD38 depletion. Thus, $CD38^+$ cells seem to actively suppress the PBMC proliferative response in certain donors, but not in others.

It was possible that the enhanced proliferative responses seen for $CD38^-$ PBMC was a result of differential time peaks for depleted compared to undepleted PBMC. To exclude this possibility I performed proliferative assays to PPD on undepleted and $CD38^-$ PBMC on days 5, 6 and 7 for five donors (Figs 3.9A and B, representative results for 2 donors). For all donors the effect of enhanced proliferative responses following CD38 depletion was maintained over the 3 days, and the peak proliferative response occurred on day 5 for undepleted and CD38 depleted PBMC.

Figure 3.9



Proliferative time course experiments indicated that the CD38 depleted PBMC (hatched bars) had higher proliferative counts compared to undepleted PBMC (black bars) over a 3 day period. Standard error bars are shown.

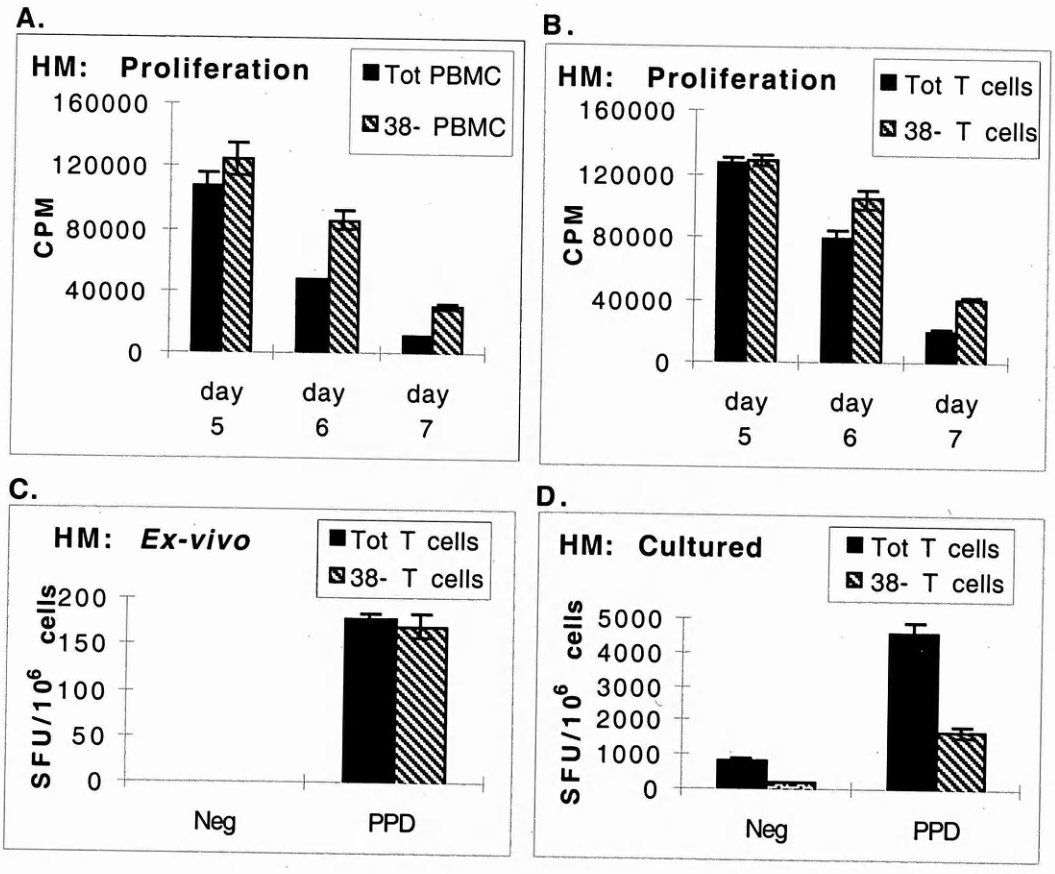
CD38 is expressed on macrophage / monocytes in addition to activated T cells (Funaro *et al.*, 1990), and hence the effect of depletion of $CD38^+$ cells might have been due to loss of suppressive antigen presenting cells (APCs). I thus purified T cells for donor HM, and assessed the effect of CD38 depletion of T cells on PPD proliferative responses. Adherent cells were irradiated and used as APCs in the T cell assays. I confirmed that the effect of CD38 depletion can occur for purified T cells (Figure 3.10B), as well as PBMC (Figure 3.10A), although this does not exclude an additional role for $CD38^+$ APCs. *Ex-vivo* ELISPOT and cultured ELISPOT responses using purified T cells and irradiated APCs at the same time led to an unchanged *ex-vivo* response (Figure 3.10C), and a diminished cultured ELISPOT response (Figures 3.10D). This result (unchanged *ex-vivo* and decreased cultured ELISPOT on CD38 depletion) was confirmed in T cell studies for 2 more donors (not shown).

3.3.8

CD38 Depletion Enhances PBMC Proliferation to a CS Protein CD4 T Cell Epitope in a Malaria Exposed Donor

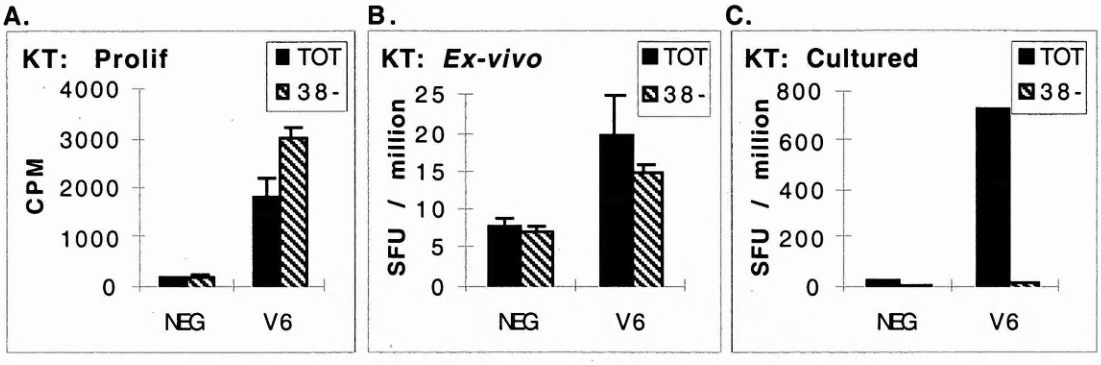
I was curious to see if the same phenomenon could be demonstrated for a malaria response, since it has long been postulated that malaria immunosuppression may be caused by an actively suppressive T cell subset. I depleted donor KT PBMC of CD38 cells, and tested responses to the immunodominant Th2R CD4 malaria epitope V6 (see Figure 3.1 for location of epitope within CS). Day 5 lymphoproliferative responses were significantly enhanced on CD38 depletion (Figure 3.11A, n = 2 experiments); *ex-vivo* ELISPOT responses were not significantly different (Figure 3.11B, n = 2 experiments); and cultured ELISPOT responses were markedly decreased (Figure 3.11C, n = 2 experiments).

Figure 3.10



PPD proliferative responses for donor HM using undepleted (black bars) and CD38 depleted (hatched bars) PBMC (A) and purifed T cells (B) confirmed that CD38 depletion led to an increased proliferation of purified T cells. The ex-vivo IFN-γ ELISPOT PPD response was unaffected by CD38 depletion of purifed T cells (C), and the cultured ELISPOT response was markedly reduced (D). Irradiated adherent PBMC were used as APCs for the purified T cells, and did not proliferate (not shown). Standard error bars are shown

Figure 3.11



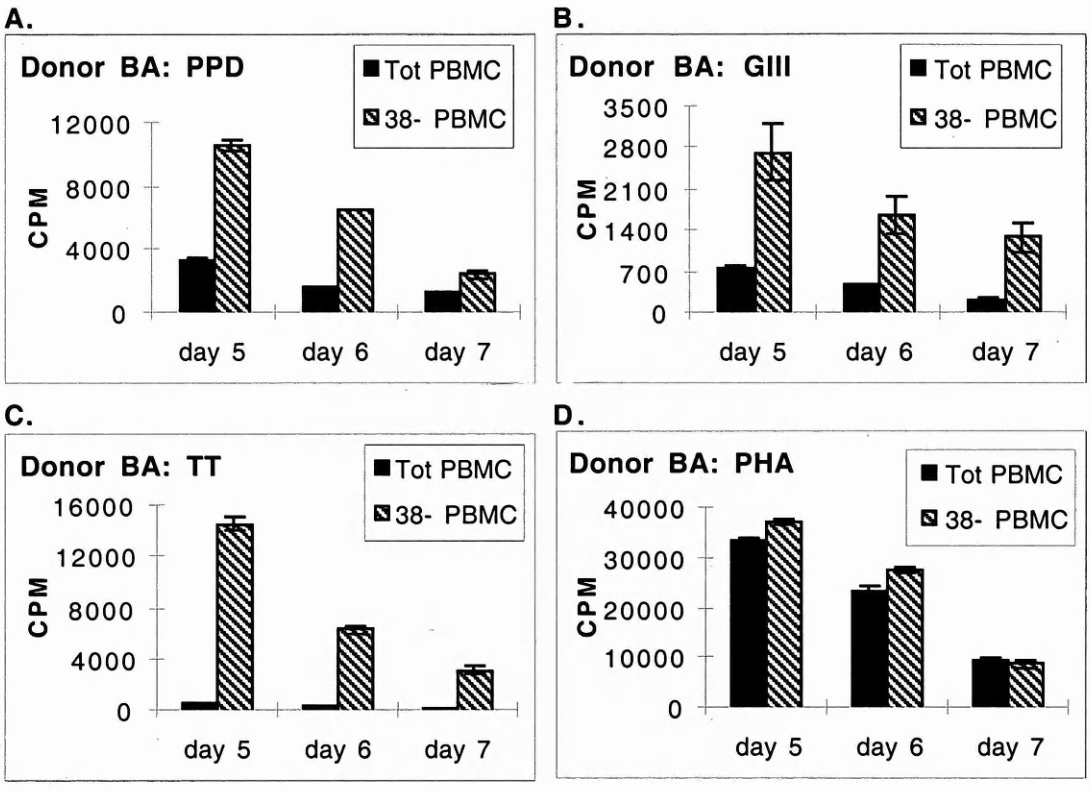
Effect of CD38 depletion on a CD4 T cell malaria epitope response (V6). Donor KT PBMC (black bars) were depleted of CD38 cells (hatched bars) and tested in proliferative assay (A, n = 2 experiments), ex-vivo IFN-γ ELISPOT (B, n = 2 experiments) and cultured IFN-γ ELISPOT (C, n = 2 experiments) for responses to peptide V6. Proliferative responses were enhanced (A), ex-vivo ELISPOT responses unchanged (B), and cultured ELISPOT responses absent(C) in the CD38 depleted wells. Standard error bars are shown.

3.3.9

$CD38^+$ T Cells Do Not Proliferate and Actively Inhibit Lymphoproliferative Responses

Having established that removal of $CD38^+$ cells from PBMC or T cells led to an enhanced proliferative response to PPD and the Th2R malaria epitope, I wanted to investigate the mechanism of this effect. In order to do this I recruited a number of healthy adult donors from the laboratory in Oxford who responded to a variety of common antigens including PPD, tetanus toxoid (TT), and a crude gluten digest (GIII), which is thought to contain a number of CD4 epitopes (R. Anderson, personal communication). CD38 depletion of PBMC was found to enhance proliferative responses to TT, a CD4 T cell response, in 2 out of 3 donors tested (Figure 3.12C), and GIII in 2 of 3 donors tested (Figure 3.12B). PHA responses were unaffected by CD38 depletion in these 3 donors (Figure 3.12D, suggesting that the effect is antigen specific.

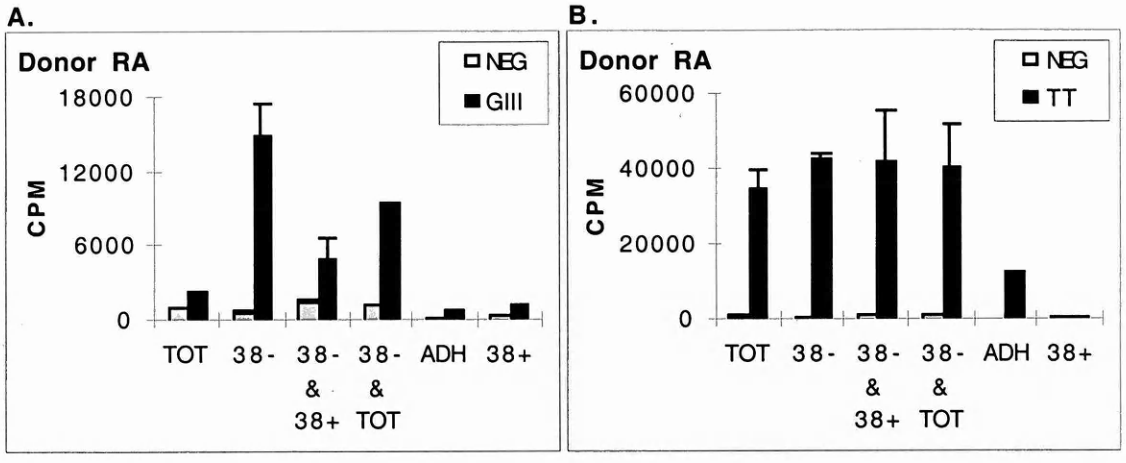
Figure 3.12



CD38 depletion (hatched bars) of PBMC (black bars) was found to effect lymphoproliferative responses to PPD (A), a gluten derived peptide digest called GIII (B), and tetanus toxoid or TT (C). There was no significant effect on the response to the mitogen PHA (D), suggesting that the response was antigen specific. Representative results for one donor. Standard error bars are shown.

I investigated whether adding back $CD38^+$ cells actively inhibits proliferation. PBMC were thus depleted of $CD38^+$ cells, and the effect of adding these back to the $CD38^-$ cell fraction was investigated. The GIII response for donor RA was actively inhibited by adding back $CD38^+$ PBMC (67% inhibition), and the $CD38^+$ cells alone did not proliferate (Figure 3.13A). The $CD38^+$ cells were added at a ratio of 1:2 38^+:38^- cells, with a constant number of $CD38^-$ cells in all wells in which they were tested. In the same donor CD38 depletion had no effect on the initial vigorous proliferative response to TT, and adding back the $CD38^+$ cells also had no effect (Figure 3.13B).

Figure 3.13

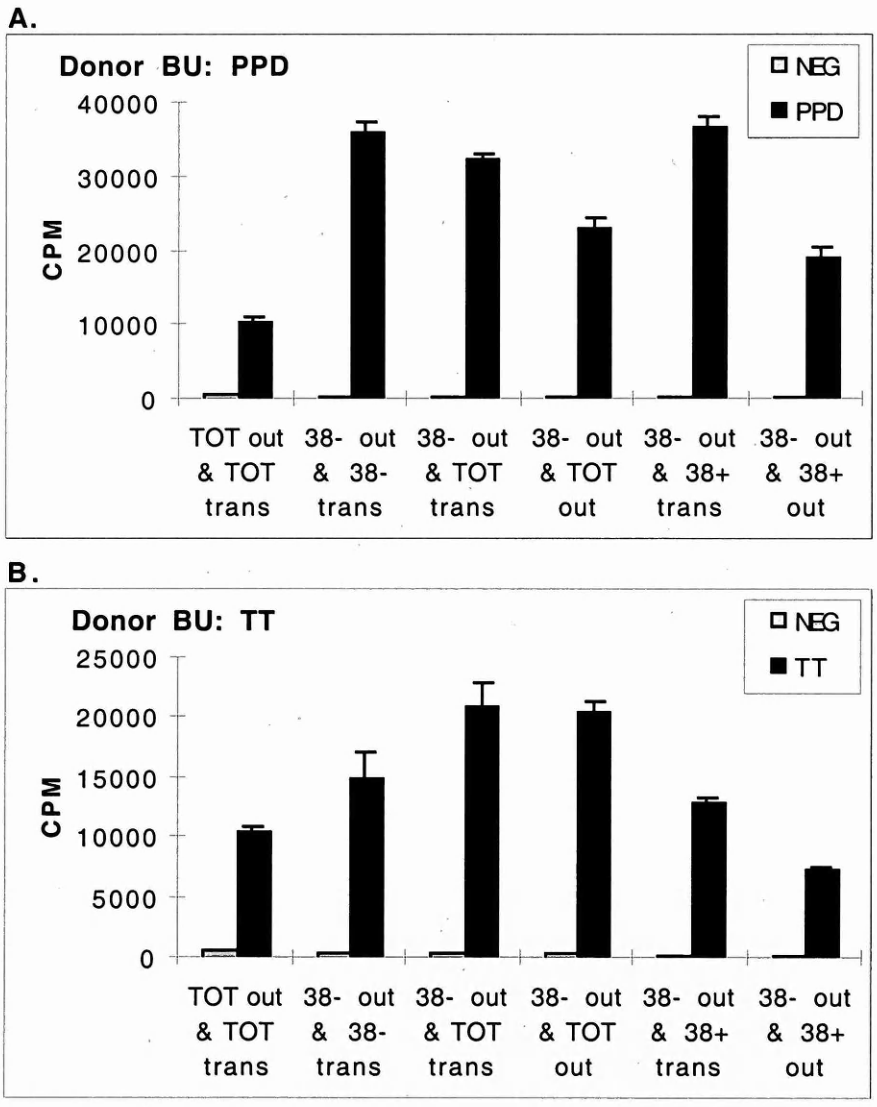


Adding CD38$^+$ cells back to the CD38$^-$ cells led to a 67% inhibition of the proliferative response to GIII for donor RA (A). Neither the irradiated adherent cells, nor the CD38$^+$ cells were able to proliferate. There was no effect of CD38 depletion on the vigorous TT proliferative response (B), and adding back CD38$^+$ cells had no effect in this case. This is consistent with the earlier observations for PPD that a high starting CPM was generally not under the influence of CD38$^+$ suppressor cells, and also shows antigen specificity for the effect. Standard error bars are shown.

This further supports our hypothesis that CD38$^+$ cells tend not to be suppressive when the initial proliferative response is high, and also shows antigen specificity. Inhibitory CD38$^+$ cells might exert their inhibitory effect either by a direct effect on the proliferating cells, or via the production of inhibitory cytokines such as IL-10 and TGF-β. In order to investigate whether direct cell-cell contact was required, or whether a soluble mediator was involved, I performed experiments with transwells. Thus, I added back CD38$^+$ cells either directly into the well containing CD38$^-$ cells, or into transwells which allow the passage of soluble inhibitory factors but no direct cell contact. I found for PPD and TT responses that there was no inhibition when 38$^+$ cells were placed in a transwell (38$^-$ out + 38$^+$ trans), but that direct addition of CD38$^+$ cells (38$^-$ out + 38$^+$ out) led to inhibition of the response (Figures 3.14A and B). Direct addition led to a 47% inhibition of the PPD response, and a 51% inhibition of the TT response. The corresponding change in counts on adding CD38$^+$

cells across a transwell were 0% for PPD and 14% for TT. Interestingly, addition of undepleted PBMC also caused an inhibitory effect of 36% for the PPD response, but had no effect on the TT response. It is possible that the $CD38^+$ fraction of the undepleted PBMC was inhibitory in the former case.

Figure 3.14

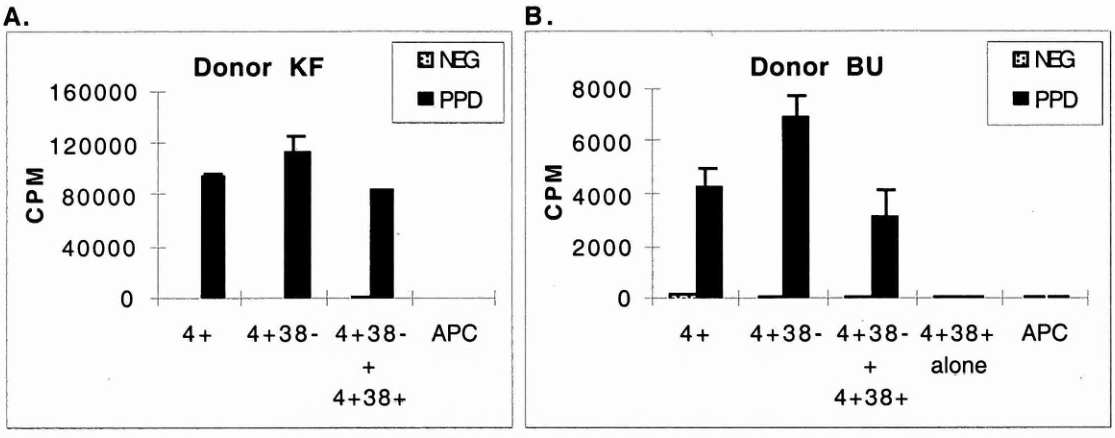


The inhibitory effect of the $CD38^+$ cells required cell-cell contact. A significant inhibitory effect was seen when $CD38^+$ cells were added directly to $CD38^-$ cells (38^- out and 38^+ out), but not when added to a transwell (38^- out and 38^+ trans). This was demonstrated for PPD (A) and TT (B) specific responses. Standard error bars are shown. Ratio of $CD38^+$:$CD38^-$ was 1:2

The above experiments were performed using PBMC, but since the responses to PPD, TT and GIII are predominantly thought to be CD4 in nature, I went on to perform experiments using purified $CD4^+$ T cells. Experiments were performed for 3 donors. Inhibition by $CD4^+38^+$ T cells was demonstrated in all 3 cases. Donor KF $CD4^+38^-$ cells proliferated vigorously to PPD, and this was inhibited by 25% when $CD4^+38^+$ cells were added to the well at a 1:1 ratio (Figure 3.15A). It is important to note that the wells containing the inhibitory cells had twice the number of cells per well compared to the $CD4^+38^-$ wells, since a constant number of $CD38^-$ cells was used for all experiments. $CD38^+$ cells at a ratio of 1:8 were able to cause 53% inhibition of a $CD4^+CD38^-$ T cell PPD response for donor BU (Figure 3.15B), which is the lowest ratio at which I have observed inhibition. Similar results were obtained for a GIII response in 1 donor with 31% suppression at a ratio of 1:4 38^+:38^- $CD4^+$ T cells. $CD4^+38^+$ cells did not proliferate in the two previous experiments.

I have therefore demonstrated that $CD38^+$ PBMC suppressed proliferation by 47%-67% in 2 donors, and $CD4^+38^+$ T cells suppressed proliferation by 25%-53% in 3 donors. The lowest ratio at which I observed suppression of proliferation was 1:8 $CD38^+$:$CD38^-$ cells. FACScan analysis for the above donors showed that 2-12% of CD4 T cells and 4-10% of CD8 T cells express CD38 on their surface, with marked variation between donors. Thus I have demonstrated inhibition of proliferation *in-vitro* at ratios of $CD38^+$:38^- CD4 T cells that exist *in-vivo*, further supporting an *in-vivo* immunosuppressive role for the $CD38^+$ population.

Figure 3.15



The enhanced proliferation following CD38 depletion (4^+38^-), and subsequent inhibition by adding back $CD38^+$ cells ($4^+38^- + 4^+38^+$), was demonstrated using purified $CD4^+$ T cells from 2 donors KF (A) and BU (B). Standard error bars are shown.

3.4

DISCUSSION

By assaying three T cell effector functions simultaneously in malaria exposed Gambians, I obtained an increased number of CS protein responsive donors than found in previous studies employing proliferation assays alone. Thus the number of individuals with T cells capable of responding to a particular antigen can be greatly underestimated unless multiple parameters of T cell activation are measured. Importantly, I show that epitope regions may be entirely missed if only proliferative assays are performed. The pattern of reactivity for each donor varied according to the effector function assessed, exposing different immunodominant regions in the carboxy-terminus of CS, and providing a broader picture of T cell reactivity for each donor.

All adults living in this region of The Gambia are repeatedly exposed to malaria throughout their life, and have developed a state of partial immunity by adulthood. Despite this, 46% of donors had low anti-CS Ab levels of <4μg/ml. I found that proliferative responses were weakly but significantly correlated with anti-CS Ab levels, a result consistent with the

findings of Hoffman and colleagues (Hoffman *et al.*, 1989b), although other studies report no such correlation (Riley *et al.*, 1990, Good *et al.*, 1988d, de Groot *et al.*, 1989). This correlation was no longer significant if only those donors with high Ab levels (>10μg/ml) were considered, and thus our result regarding correlation is tentative. The *ex-vivo* and cultured IFN-γ ELISPOT responses did not correlate with Ab levels, nor was a correlation between Abs and IL-4 ELISPOT responses to CS protein seen in Gambian adults. The latter differs from the findings of Troye-Blomberg and colleagues who reported a correlation between IL-4 production and elevated serum Ab levels to peptides from the blood stage Ag *Pf*155 / RESA in a study of 8 adult Gambian donors (Troye-Blomberg *et al.*, 1990).

Thirty five percent (17/48) of naturally exposed donors in this study gave a T cell response with a concurrent low Ab response (<4μg/ml) suggesting that T cell functions other than help in Ab production play a role in natural immunity to malaria. A further ten percent (5/48) of the donors in this study had low Ab levels and no peptide response in any of the 3 assays. Such T cell nonresponsiveness is characteristic of natural immunity to malaria antigens, both in parasitaemic and aparasitaemic individuals (Good *et al.*, 1988d, Riley *et al.*, 1988b, Troye-Blomberg *et al.*, 1989, Hviid *et al.*, 1990), the possible causes for which are reviewed in Chapter 1.

There was no correlation between positive responses in any of the three T cell assays, suggesting that each assay detects a distinct functional T cell subset. Depletion studies confirmed that IFN-γ *ex-vivo* ELISPOT and proliferative responses were mediated by $CD45RA^{-}RO^{+}$ cells (Mackay, 1993). CD45RB expression has been shown to define a T cell subset within the $CD45RO^{+}$ memory T cell subset that is capable of IFN-γ secretion (Swain, 1991), and indeed depletion of CD45RB cells led to a significant decline in IFN-γ ELISPOT responses to PPD, but had a variable effect on lymphoproliferation.

Recently, human memory $CD45RO^{+}$ T cells have been divided into two functionally distinct subsets according to the presence or absence of expression of the chemokine

receptor CCR7 (Sallusto *et al.*, 1999). Depletion of cells expressing the chemokine receptor CCR7 showed that cultured ELISPOT responses require the presence of $CCR7^+$ cells, whereas *ex-vivo* ELISPOT and lymphoproliferative responses do not. I thus have preliminary evidence in 4 donors supporting our hypothesis that the *ex-vivo* ELISPOT detects $CCR7^-$ circulating effector T cells (T_{EM} cells) in response to antigen, whilst the cultured ELISPOT detects a $CCR7^+$ central memory population (T_{CM} cells). CCR7 controls homing to secondary lymphoid organs, and its ligand is expressed on endothelial cells. $CCR7^+$ cells home to lymph nodes, whereas $CCR7^-$ cells express receptors for migration to inflamed tissues.

Depletion of cells expressing the T cell activation marker CD38, led to increased lymphoproliferative responses to PPD, with either decreased or unchanged IFN-γ ELISPOT responses. This effect on lymphoproliferation was dependant on the original proliferative response in the undepleted PBMC. Thus, only those donors with low to moderate counts to PPD prior to depletion, had enhanced counts following depletion. This phenomenon was highly correlated for 14 donors, and those with the lowest starting counts had the greatest relative increase following depletion. The enhancement of responses following CD38 depletion was shown to be mediated by a direct suppressive effect of $CD38^+$ PBMC in 2 donors, and more specifically $CD4^+38^+$ T cells in 3 donors. A CD4 T cell malaria epitope proliferative response was also enhanced by CD38 depletion, confirming the relevance of this mechanism of T cell immunosuppression to malaria. My findings are in keeping with previously published data in the murine model showing that a population of $CD4^+38^-$ T cells mounts a vigorous proliferative response, but that the presence of a $CD38^+$ population significantly inhibits anti-CD3 induced proliferation (Read *et al.*, 1998).

CD38 is a type II transmembrane glycoprotein with catalytic activity, but largely unknown function, expressed by different immunocompetent cells including activated T cells (Funaro *et al.*, 1990, Lund *et al.*, 1998). Like CCR7, its natural ligand is an endothelial receptor, in this case CD31, and CD38 is thought to play a role in endothelial adhesion of

activated T cells. Human CD38 expression is increased in a variety of pathological conditions such as myeloma and lymphoma. An association between high levels of CD38 expression on $CD8^+$ T cells and poor prognosis in HIV infection is also well established (Giorgi *et al.*, 1993, Liu *et al.*, 1997), although the basis of this association is unknown. The immunoregulatory effect that I describe could play a role, since high CD38 expression may lead to a decreased proliferative capacity in response to the virus. CD38 expression has not been investigated in malaria, but a putative role in the poor lymphoproliferative responses that characterise natural immunity in malaria exposed donors is attractive. Understanding the differential immunoregulatory role of human $CD38^+$ T cells may be important in the development of vaccines for malaria, and indeed other infectious diseases where proliferative and IFN-γ responses play a protective role such as tuberculosis (Torres *et al.*, 1994, Sinha *et al.*, 1997) and leishmaniasis (Haberer *et al.*, 1998).

In summary, I have provided evidence that the currently accepted classification of memory T cells is incomplete. It is likely that they can be further subdivided, each group with a unique role in defence against disease. I have confirmed that $CCR7^+$ cells are necessary to generate a cultured ELISPOT response, but are not required for *ex-vivo* ELISPOT responses or lymphoproliferation. I have also demonstrated that regulatory $CD4^+38^+$ T cells suppress lymphoproliferative responses to several CD4 antigens. Further assessment for T cells expressing various chemokine receptors (eg. CXCR3, CXCR4 and CCR5) (Sallusto *et al.*, 1998), or cell surface markers (CD27, CD148) (Baars *et al.*, 1995, Tangye *et al.*, 1998) may well lead to further subcategorisation of memory T cells. The precise role of these T cell subsets in protection against malaria, and indeed other infectious diseases, remains to be determined, with the exciting prospect that understanding these important issues will facilitate development of a new generation of immunoregulatory vaccines.

CHAPTER 4

ALTERED PEPTIDE LIGAND ANTAGONISM FOR A VARIANT CD4 T CELL EPITOPE OF CS PROTEIN

4.1

INTRODUCTION

I demonstrated in chapter 3 that the number of donors responding to CS protein derived peptides can be greatly enhanced by employing 3 T cell assays simultaneously (*ex-vivo* IFN-γ ELISPOT, cultured IFN-γ ELISPOT and lymphoproliferation). The 15mer epitopes used for this study were derived from of two allelic variants of CS (clone NF54 and 7G8). However, the carboxy-terminus of CS, which contains most of the T cell epitope regions, is highly polymorphic. Individuals living in malaria endemic regions may expect to see multiple CS variants throughout a lifetime of parasite exposure, with over 10 polymorphic variants of particular epitopes present in a region at any one time.

The highest level of variation is found in two immunodominant proliferation inducing CD4 T cell epitope regions within the carboxy-terminus of CS, denoted Th2R (aa 326-347) and Th3R (aa 361-380) (see Figure 3.1). CD8 T cell epitopes also exist within these regions, and are named Tc2R (aa 327-335) and Tc3R (aa 369-375) respectively. Exclusively nonsynonymous nucleotide changes occur in these epitope regions suggesting that they are under significant immune selection pressure, and that polymorphism is maintained as an immune escape strategy employed by the parasite. However, it has not been determined how CD4 T cells exert such selective pressure on the malaria parasite.

Adult Gambians receive an average of five bites from malaria infected mosquitoes every year, and by adulthood will have received in the order of 100 infectious bites (Greenwood *et al.*, 1987, Gupta *et al.*, 1994). Despite this continual antigenic exposure, many Gambian donors fail to respond to CS protein in proliferation assays (Good *et al.*, 1988d, Good *et al.*, 1988b), even to the universally binding variant of the Th2R epitope (Calvo-Calle *et al.*,

1997). Epitope polymorphism is not responsible for this low reactivity since lymphoproliferative responses to polymorphic variants of the Th2R and Th3R CD4 epitope regions are generally non-crossreactive (de la Cruz *et al.*, 1988a, de la Cruz *et al.*, 1989, Udhayakumar *et al.*, 1994, Zevering *et al.*, 1994). Again this is curious given that adults will have been exposed to each of the variants during their lifetime, and might be expected to respond to all variants. A generalised immunosuppression is unlikely to explain these phenomena since responses to other recall antigens such as PPD are found at the expected frequencies (Ho *et al.*, 1986). I hypothesised that perhaps more subtle immune evasion strategies are at play here.

Gilbert, Plebanski and colleagues found that an HLA-B35 restricted CD8 variant T cell epitope (Tc3R derived) can lead to impairment of the CTL activity to a second variant of the same epitope region by a process known as altered peptide ligand antagonism (APL) (Gilbert *et al.*, 1998). Thus, the variant denoted cp26 switches off CTL responses to cp29, and in fact cp29 can in turn antagonise cp26. The same authors demonstrate that cp26 and cp29 can mutually interfere with the induction of primary CTL responses *in vitro*. They further demonstrate that APL leads to a skewed parasite distribution of the 2 strains in naturally exposed Gambians (Gilbert *et al.*, 1998), thus suggesting the *in vivo* relevance of this phenomenon. Antagonism has not however been studied for any CD4 CS T cell epitope regions, nor indeed for a CD4 T cell response to any infectious pathogen.

4.2

STUDY DESIGN

4.2.1

Rationale

I first aimed to assess whether polymorphism in the immunodominant Th2R CD4 T cell epitope region generates variants with little cross-reactivity. I performed IFN-γ ELISPOT assays using PBMC from adult Gambians to 9 naturally occurring Th2R variants present in The Gambia (Table 4.1). Given our evidence in the previous chapters that this assay detects effector memory T cells (T_{EM}) in response to recent exposure (e.g. up to 2

months), it might be particularly useful for assessing true cross-reactivity, as opposed to cross-recognition of multiple variants, since each donor is unlikely to have been exposed to multiple strains in the preceding few months. I then addressed whether APL within the Th2R CD4 T cell epitope region might play a role in the maintenance of natural polymorphism within this region.

4.2.2

Study Site and Volunteers

Healthy adult informed consent volunteers were recruited from the villages of Brefet, Dampha Kunda and Bakau in The Gambia (Figure 3.2). Malaria exposed adults working at the John Radcliffe Hospital, Oxford were also recruited. A further 20 healthy adults who had never been exposed to malaria were recruited from the John Radcliffe Hospital in Oxford, and used as naïve controls.

4.3

RESULTS

4.3.1

IFN-γ ELISPOT Responses to Variants of the Th2R CD4 Epitope Region Are Found in Less Than Half of Naturally Exposed Adult Gambians and Show Limited Cross-Reactivity

The Th2R CD4 T cell epitope region of CS has at least 9 naturally occurring variants in The Gambia, and the Th3R CD4 epitope region has a minimum of 8 (de la Cruz *et al.*, 1987, Good *et al.*, 1988d, Lockyer *et al.*, 1989). Variant V6 of the Th2R region (see Table 4.1) exhibits a broad spectrum of HLA-DR binding (Calvo-Calle *et al.*, 1997). The amino acid variants arise at positions 329, 332, 333, 336, 337, 339 and 342, 5 of which are dimorphic, and 2 are trimorphic (Table 4.1). I first studied the binding potential of all 9 variants of this region to three common African alleles (HLA-DRB1*1302, HLA-DRB1*0101, and HLA-DRB1*1501) using an *in vitro* binding assay (Davenport *et al.*, 1995b) (see Methods). All variants bound to HLA-DRB1*1501, and 8/9 to HLA-DRB1*1302 and HLA-DRB1*0101 (IC50<100μg/ml) (Table 4.1).

Table 4.1

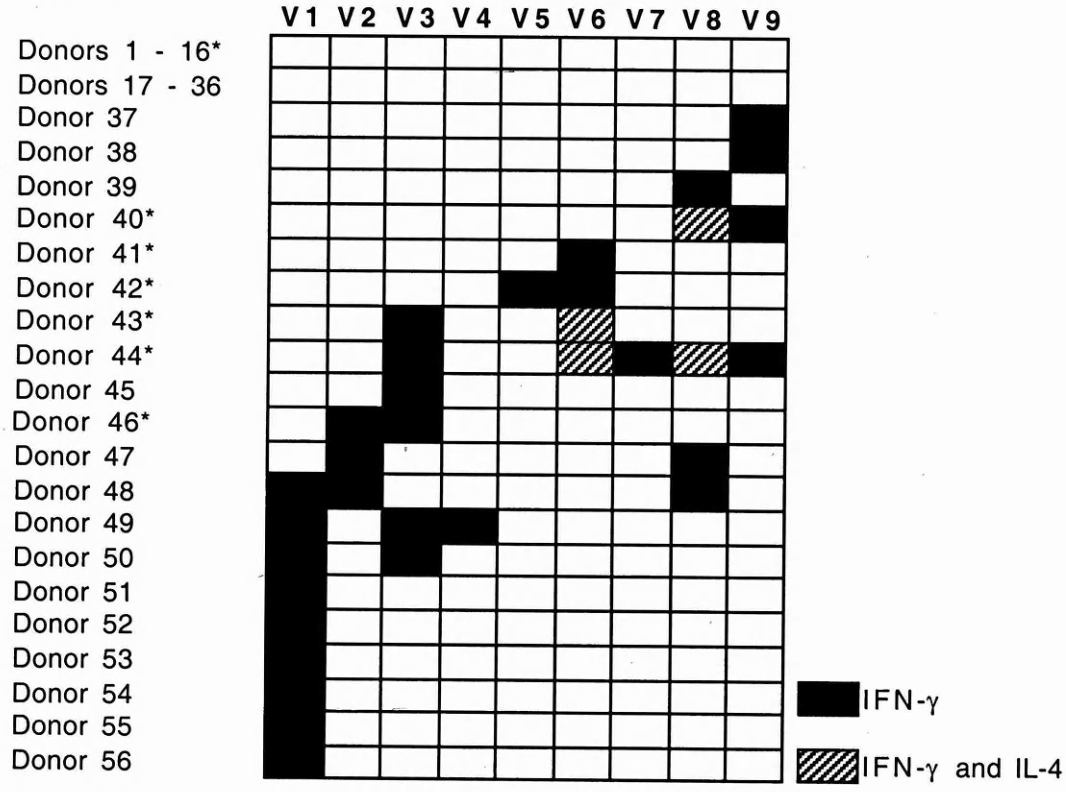
VARIANT	SEQUENCE	DR1302	DR1	DR15
V1	PSD**Q**HI**E**K**K**YL**K**T**K**I**Q**NS**L**STEWS	+ +	+ +	+ +
V2	PSD**Q**HI**E**K**K**YL**K**T**K**I**K**NS**L**STEWS	+ +	+ +	+
V3	PSD**Q**HI**E**K**K**YL**Q**K**K**I**Q**NS**L**STEWS	+ +	+ +	+ + +
V4	PSD**Q**HI**E**K**K**YL**Q**K**K**I**K**NS**L**STEWS	+ +	+ +	+
V5	PSD**Q**HI**E**K**K**YL**Q**K**K**I**R**NS**L**STEWS	+ +	+ + +	+ +
V6	PSD**K**HI**K**E**K**YL**N**K**K**I**Q**NS**L**STEWS	+ + +	+ +	+ +
V7	PSD**K**HI**E**Q**K**YL**N**T**K**I**Q**NS**L**STEWS	+ +	+ +	+ +
V8	PSD**K**HI**E**Q**K**YL**K**K**K**I**Q**NS**L**STEWS	-	-	+ + +
V9	PSD**K**HI**E**Q**K**YL**K**K**K**I**K**NS**I**STEWS	+ +	+ + +	+ +
pCLIP		+ +	+ +	ND
pTT		+ +	ND	+ +

Binding of natural variant peptides of the Th2R CD4 T cell epitope region of CS protein. Polymorphic positions are marked in bold. Peptides were scored as high (+++), medium (++) and low (+), or undetectable (-) binding capacity (IC50 <1, 1-25, 25-100, >100μg/ml respectively) to each HLA-DR allele tested. The universally binding epitopes from TT (pTT) and CLIP (pCLIP) were used as positive controls. ND = not determined.

Early IFN-γ release from freshly isolated PBMC, to the 9 Gambian variants of the Th2R region, was assessed by ELISPOT (Table 4.2A). Of 56 healthy adult Gambians tested, only 20 (36%) responded to any Th2R peptide despite the fact that all donors had a lifetime of malaria exposure (Table 4.2). In only 9 donors (16%) were more than one variant peptide recognised, suggesting remarkable specificity when an IFN-γ ELISPOT response occurred. Indeed, all amino acid changes in these variant epitopes could lead to lack of cross-recognition in these individuals. Many infections in The Gambia are known to be co-infections of two or more strains of CS (Gilbert *et al.*, 1998), and I postulated that non-responders for IFN-γ, a characteristic Th1 type cytokine, might have generated a Th2 type cytokine response characterised by IL-4 production instead (Mosmann and Coffman,

1989). I thus measured stimulation of early IL-4 production in parallel with IFN-γ in 22 of the donors. Only 3 of the 22 donors gave an IL-4 response, and all of them produced IFN-γ to the same variant (Table 4.2A). None of the 20 malaria naïve donors tested reacted to any of the variants in identical early lymphokine release assays.

Table 4.2

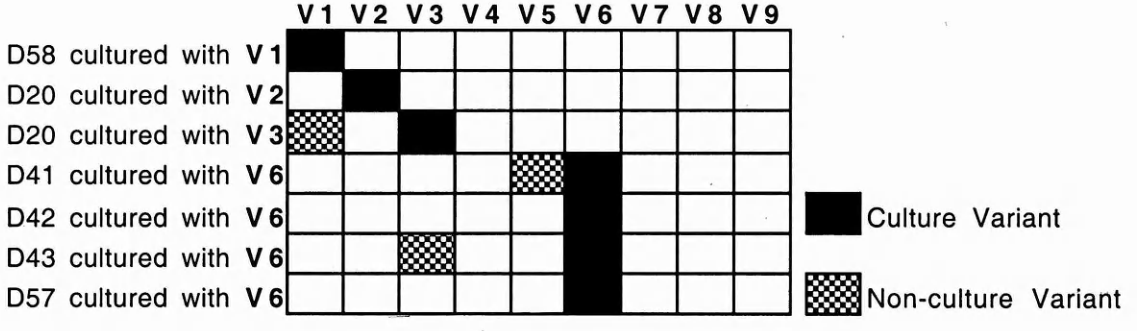


* tested for IFN-γ & IL-4

Limited cross-reactivity was observed by IFN-γ ex-vivo ELISPOT testing with 9 naturally occurring polymorphic variants of the Th2R CD4 T cell epitope region of CS protein. 22 of the individuals (marked with an asterisk) were tested in parallel for IL-4 reactivity by overnight ELISPOT. The hatched squares indicate where both IFN-γ and IL-4 responses were detected to the same variant epitope in the same donor. 16 of the donors gave neither IFN-γ nor IL-4 responses (D1-16 at top of table), and no donors produced IL-4 alone. 20 malaria naïve donors were tested for reactivity to the same variants but failed to react to any of them, but gave the expected frequencies to control antigens PPD and TT and to the mitogen PHA (data not shown).

Cross-reactivity was further investigated by restimulating *in vitro* with a single Th2R variant peptide, and testing for cross-reactivity against all variants after 14 days of expansion in culture (Table 4.3). PBMC from malaria-exposed Gambians (Donors 20, 41, 42, and 43) and malaria-exposed Europeans (Donors 57 and 58) were cultured separately with single variants, and then each cell line was tested for reactivity against all the other variants by IFN-γ ELISPOT. All responses detectable after culture were either monospecific to the culture variant, or recognised only 2 of the 9 variants (Table 4.3). Thus cross-reactive responses cannot be generated even after a 14 day period of *in vitro* culture.

Table 4.3



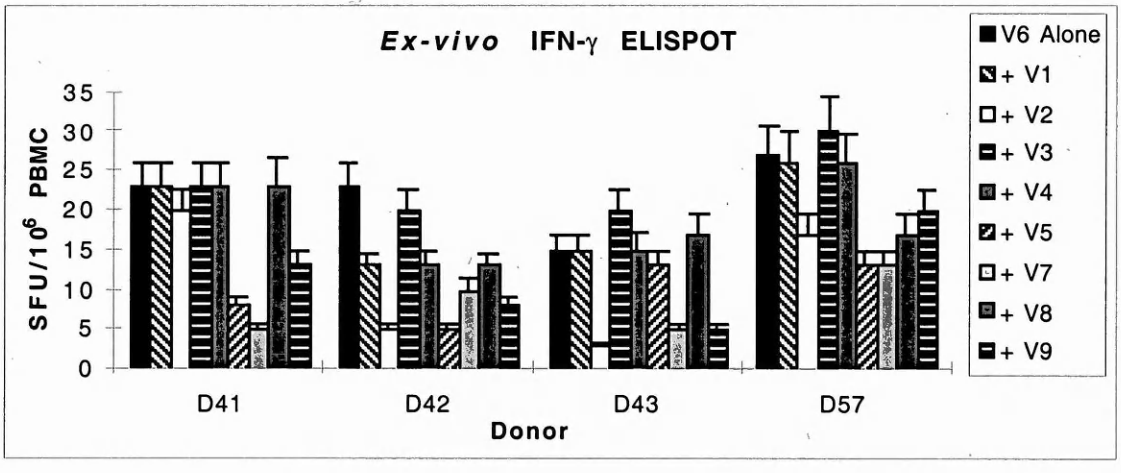
PBMC were cultured with the stated variant indicated in bold and tested 14 days later for cross-reactivity to all the 9 variants by overnight IFN-γ ELISPOT assay. Two naturally malaria-exposed European donors who reacted specifically to variants V1 (D58) and V6 (D57) in overnight IFN-γ ELISPOT assays, were also tested to evaluate the specificity of V1 or V6 stimulated short term T cell lines. All donors gave significant responses to the peptides in which they were cultured (black squares), and only rarely was a response detected to a non-culture variant (chequered squares). Donors D41, D42 and D43 gave significant responses to V6 by ex-vivo IFN-γ ELISPOT assay, whereas donor D20 responses to V2 and V3 were undetectable prior to in vitro expansion.

4.3.2

Altered Peptide Ligand Antagonism to Th2R CD4 Epitope Variants of CS Protein

Four of the 56 adult Gambians (7%) responded to the "universally binding" variant V6 of CS protein (Tables 4.1 and 4.2) (Calvo-Calle *et al.*, 1997). PBMC from three of these Gambians, and one other naturally exposed individual who responded to variant V6, were tested in early IFN-γ secretion assays to assess the effect of the presence of each one of the other 8 variants on the V6 response (Figure 4.1). These variants were added after binding with a suboptimal dose of V6 had occurred in a standard antagonism assay (De Magistris *et al*, 1992) (see Methods). Responses to V6 could be partially inhibited by co-culture with certain of the naturally occurring Th2R variants in all 4 donors studied (Figure 4.1). Surprisingly, the same variants could be inhibitory in different individuals (V2, V5, V7 and V9 in 2/4, 3/4, 4/4, and 2/4 respectively showed >50% inhibition).

Figure 4.1



Donors D41, D42, D43 and D57 respond to the V6 CD4 T cell epitope by IFN-γ secretion. The impact of the presence of the other variants on the V6 response was assessed in an antagonism assay. Index peptide was prepulsed onto PBMC at 25μg/ml, and the other variants were added 1 hour later at 50μg/ml. The assay was then run as a standard ex-vivo IFN-γ ELISPOT assay. For donor D43 the antagonism assay was repeated another 2 times with similar results, and another 12 times for donor D57. Inhibition of donor D57 V6 responses was observed from 5μg/ml of V5 or V7 (n = 2 experiments) (data not shown).

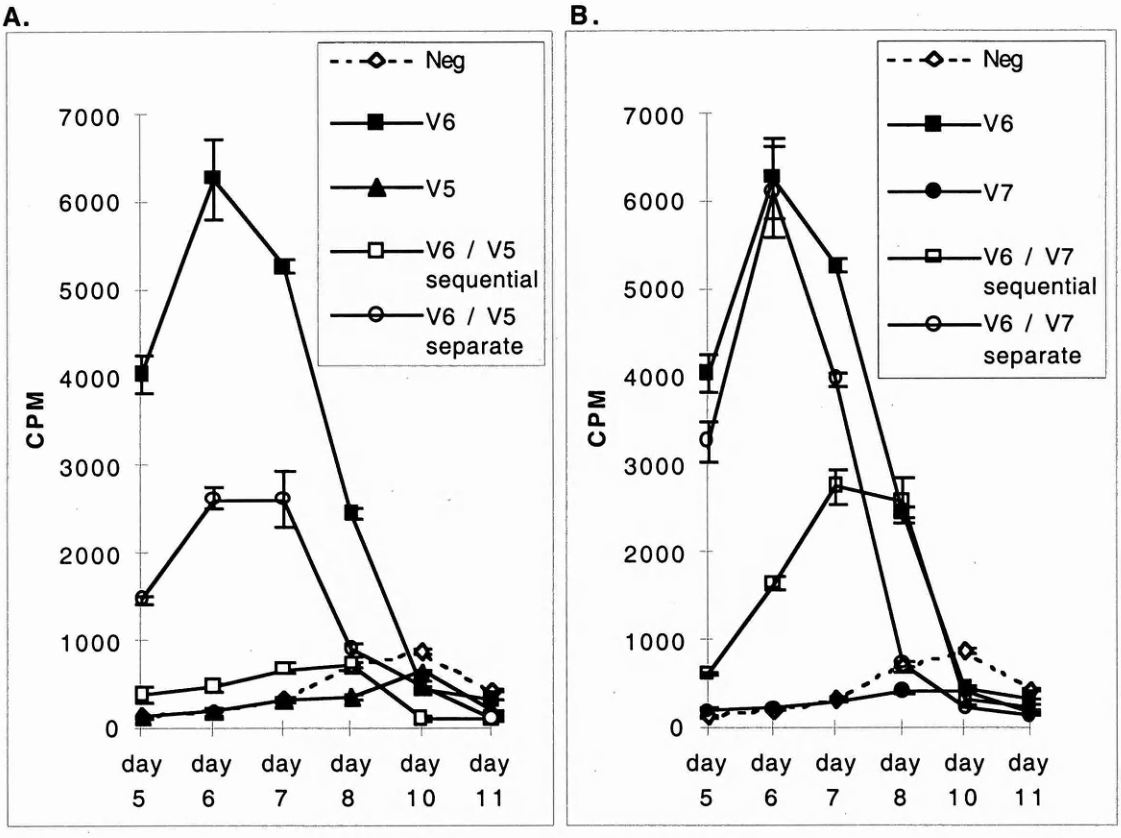
This putative antagonistic altered peptide ligand effect was investigated in detail for donor D57. The response for D57 was specific for V6 (Table 4.3). Over a 12 month period when IFN-γ antagonism assays were performed using D57 PBMC, V5 and V7 were usually inhibitory (V5 in 11 / 12 experiments, and V7 in 11 / 13 experiments) despite some variation in the absolute level of inhibition (mean inhibition for V5 39% ± 7 SE, $p < 0.0005$; V7 39% ± 9 SE, $p < 0.0009$). The universally binding HLA-DR peptide from tetanus toxoid (pTT) did not inhibit V6 responses when used in antagonism assays (n = 3), and PPD responses were not inhibited by V5, V6 or V7 (n = 2) (not shown). Figures 4.2A and 4.2B show a representative assay for donor D57 in which PBMC proliferative responses induced by V6 were strongly inhibited by V5 and V7 (n = 6 assays). Significant inhibition by V5 but not V7 could be found even if the variant epitope was presented on a different antigen presenting cell (APC) from V6 (Figure 4.2A). This *trans*-inhibition by V5 suggested the possibility of a soluble suppressive mediator.

4.3.3

Selective Induction of IL-10 by Th2R CD4 T Cell Epitope Variants

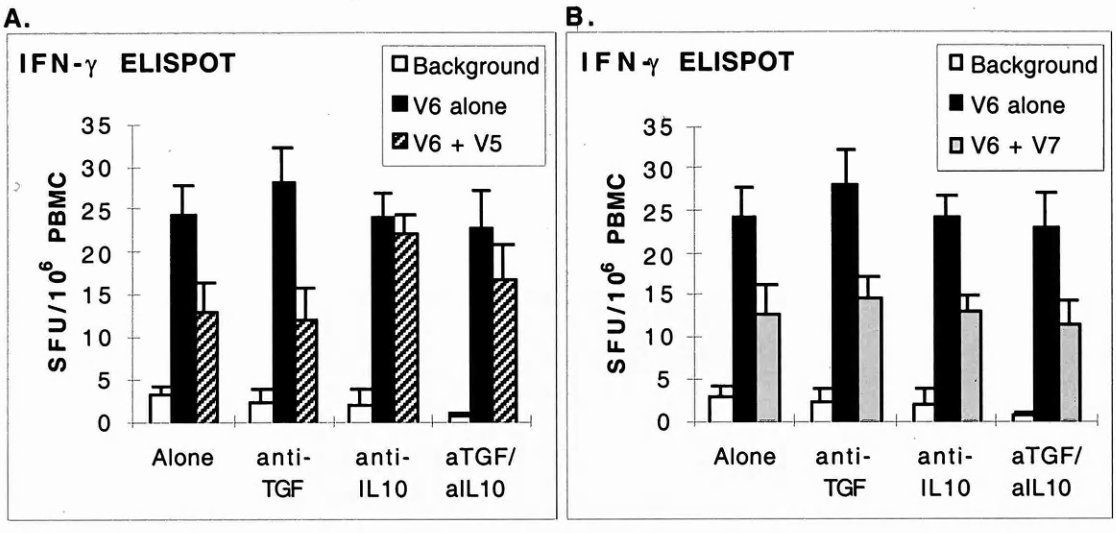
Both TGF-β and IL-10 have been shown to have suppressive effects on proliferation and cytokine production (Moore *et al.*, 1993, Groux *et al.*, 1997), and thus were potential candidate mediators for the suppressive effect of V5. Indeed, inhibition of V6 induced early IFN-γ responses by V5 could be completely reversed by neutralising anti-IL-10 but not anti-TGFβ monoclonal Abs (n = 3 assays). (Figure 4.3A). This was not the case for V7 induced inhibition, which could not be reversed by either anti-IL-10 or anti-TGFβ (Figure 4.3B). Early IL-10 production by donor D57 PBMC was assessed in ELISPOT assay in response to all 9 variant peptides (at 50μg/ml) either alone or in the presence of variant V6 (at 25 μg/ml) (Figure 4.4). Variants V5 and V7 induced IL-10 both alone, and in the presence of V6, whereas V9 induced IL-10 only in the presence of V6. Titration of responses ranging from 5-100 μg/ml showed maximal IL-10 induction by V5 and V7 at 25-50 μg/ml (not shown). V5 and V7 alone failed to stimulate significant IFN-γ or IL-4 production at any concentration tested (5-100 μg/ml, n = 6 assays) (not shown).

Figure 4.2



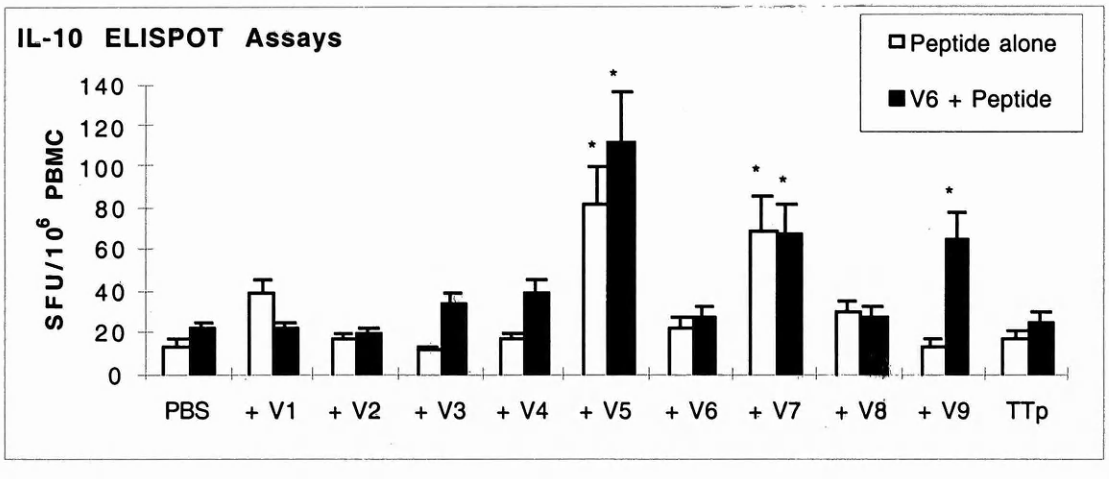
Donor D57 PBMC proliferated in response to peptide V6 (closed squares), but failed to respond to variants V5 (A, closed triangles) and V7 (B, closed circles) in parallel assays. Antagonism of the V6 specific proliferative response was induced in 2 ways. Irradiated PBMC were either sequentially prepulsed (open squares) with V6 followed by the putative antagonist, or separately prepulsed with V6 and antagonist and mixed at a 1:1 ratio (open circles). These were then washed, mixed at a 1:1 ratio with PBMC, and set up in standard proliferation assays. Tritiated thymidine incorporation was monitored from days 5-10. A representative experiment of 6 is shown. Results are shown as mean triplicate CPM ±SE

Figure 4.3



ELISPOTs were set up for antagonism assays of V5 on V6 (A) and V7 on V6 (B). Neutralising mAbs to TGFβ (10ng/ml) and IL-10 (10ng/ml), or both were added at the same time as the index peptide. Anti-IL-10 reversed the inhibitory effect of V6 (A) but not V7 (B), and anti-TGFβ had no effect even when increased to a dose of 100ng/ml (n = 2 assays, data not shown). A representative experiment of 3 is shown.

Figure 4.4

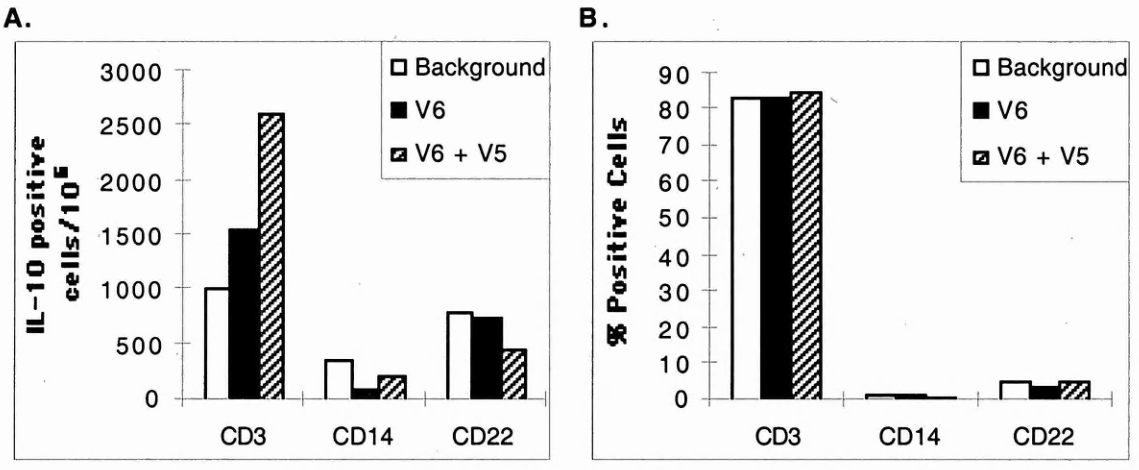


Early IL-10 production was tested by ELISPOT for donor D57 to all the Th2R variants alone (open bars) or in V6 antagonism assays (black bars). Variants were used at 50μg/ml, and significant IL-10 production over control is indicated with an asterisk ($p < 0.05$). Titration of responses (5-100μg/ml) showed maximal IL-10 induction by V5, V6 and V7 at 25-50μg/ml, either alone or in antagonism assays (n = 2, data not shown).

The effect of the V5 variant on lymphokine production by V6-specific T cells was further studied utilising lines and clones generated from donor D57. Assessment of intracellular IL-10 content of a V6 specific line (antagonisable by V5) from D57 showed doubling in number of IL-10 producing $CD3^+$ T cells by V6/V5 co-stimulation compared to background or V6 alone (Figures 4.5A and B). ELISPOT assays conducted in parallel at the time of this analysis showed 100% inhibition of the V6 IFN-γ response by V5 (not shown). The IL-10 positive cell number was unchanged for monocyte/macrophages ($CD14^+$ cells), or activated B cells ($CD22^+$ cells) (Figures 4.5A and B). Stimulation of V6-specific clones with V6 induced either IFN-γ alone (clones C2, C5 and C6) or IFN-γ and IL-10 (clones C3 and C4) secretion (Figure 4.6A and B). Stimulation of these clones with V6 in the presence of V5 led to a downregulation of IFN-γ production (Figure 4.6A) and a concomitant increase in IL-10 for all clones (Figure 4.6B). Thus, IL-10 production in CD4 T cell clones may be increased by co-stimulation with an altered peptide ligand. Oligoclonal responses (when clones were mixed) reflected the dominant change in IFN-γ / IL-10 ratio toward IL-10 production when stimulated by V6 in the presence of V5, but no upregulation of IL-10 in the presence of V7 (Figures 4.6C and D). No IL-4 could be detected under any experimental condition in parallel ELISPOT assays (not shown).

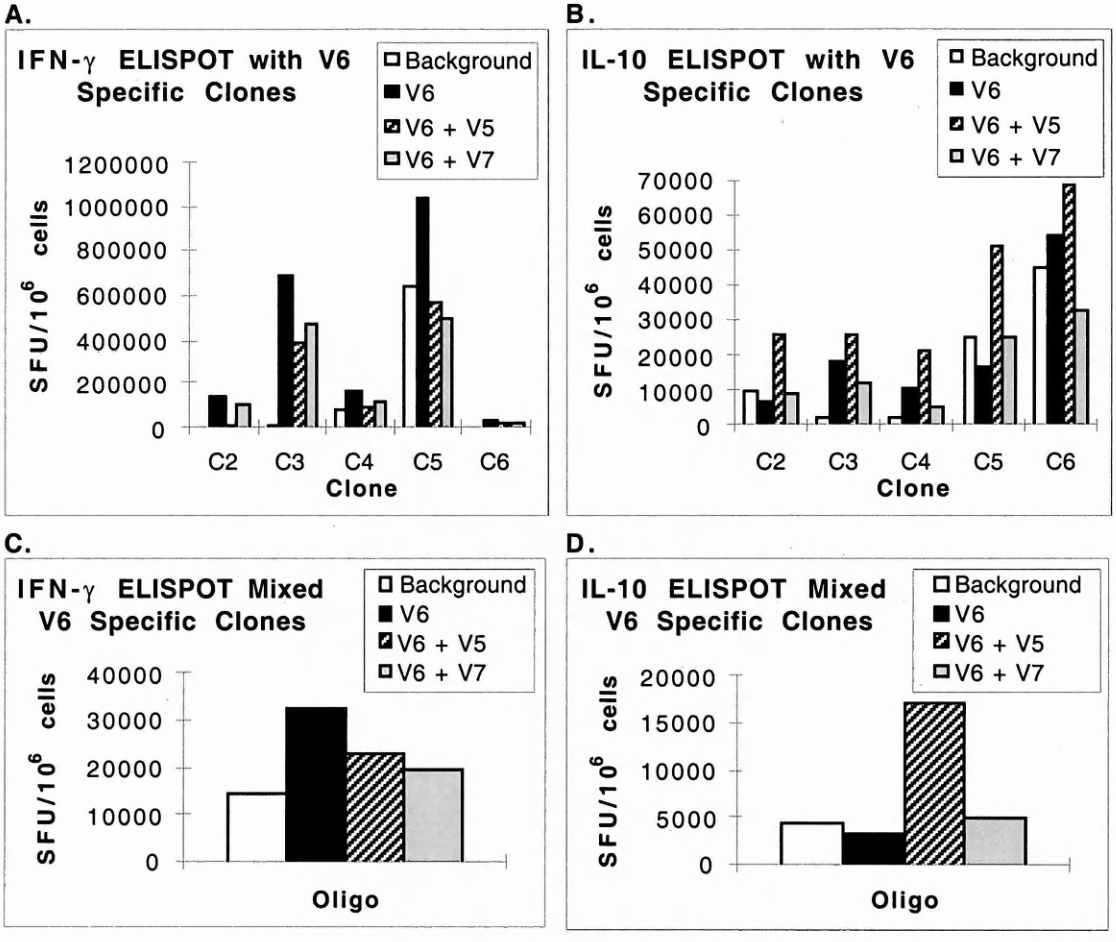
I found that clones C3 and C4 use a different TCR (not shown), and that their reactivity pattern to V5, V6 and V7 was different. Thus, clone C4 failed to produce IL-10 to V5 and V7, but still showed significant IL-10 induction by V6-V5 or V6-V7 co-stimulation (Figure 4.7A). By contrast, clone C3 responded to each of V6, V5 and V7 alone by IL-10 secretion. As expected, both clones responded by IFN-γ secretion to V6, but not to V5 or V7 alone (not shown). Fresh PBMC responses behaved more similarly in reactivity to clone C3, with IL-10 usually produced to both V5 and V7, although not to V6 (Figure 4.7B).

Figure 4.5



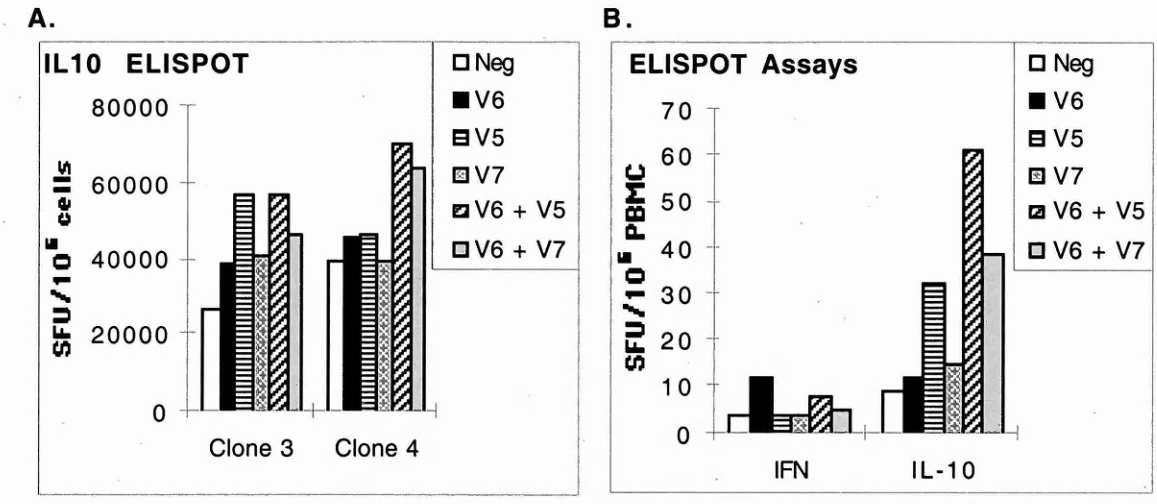
A V6-specific CD4 T cell line from donor D57 was stimulated with HLA-DR15-matched PBMC alone (white bars), or with V6 (20μg/ml) (black bars), or V6 then V5 (40μg/ml) (hatched bars). The phenotype of IL-10 positive cells was analysed by FACScan (CD3 for T cells, CD14 for monocytes/macrophages, CD22 for activated B cells). The data is presented as IL-10 positive cells per million (A) and percent CD3, CD14 and CD22 positive cells (B). The protocol resulted in 100% inhibition of the V6 IFN-γ response by V5 in an ELISPOT assay conducted in parallel with the same samples (data not shown).

Figure 4.6



V6-specific T cell clones generated from donor D57 were tested in ELISPOT antagonism assays. IFN-γ (A), IL-4 (data not shown) and IL-10 (B) production were assessed in parallel. Clones C2, C3, C4, C5 and C6 were utilised at 500 cells/well with either irradiated HLA-matched PBMC or an autologous Bcl line (at 50,000/well) as presenters. Oligoclonal responses were assessed by mixing all clones (C and D). Results shown are from single experiments performed in duplicate and are representative of four separate experiments for clones C3 and C4, two for C5 and C6, and one for C2. No significant IL-4 production was detected in any of the experiments.

Figure 4.7



Donor D57 clones C3 and C4 were further tested for their ability to respond by IL-10 production to V5 or V7 alone, compared to V5 and V7 in the presence of V6. Clone C3 showed weak IL-10 reactivity over background to V6 or V7 alone or V6+V7 ($p<0.05$) and strong reactivity to V5 alone or V6+V5 ($p<0.001$). By contrast, clone C4 failed to respond significantly to V5, V6, or V7, but responded well to V6+V5 and V6+V7 ($p < 0.05$). Both clones responded to V6 but not V5 or V7 by ex-vivo IFN-γ ELISPOT (data not shown). The results shown are from a single experiment performed in triplicate. Freshly isolated PBMC from donor D57 were set up in parallel agonism and antagonism IFN-γ and IL-10 ELISPOT assays (B). One of 2 representative assays is shown.

4.4

DISCUSSION

The family of nine naturally occurring Th2R CD4 T cell epitope variants tested here showed a remarkable lack of cross-reactivity in IFN-γ assays. This is in keeping with the findings of others, and suggested that T cell responses may not be detected unless all variants are tested. It also means that CD4 T cell responses of donors living in malaria endemic regions should not be susceptible to heterologous boosting, thus limiting their persistence *in vivo*. This lack of cross-reactivity presents major problems for the design of vaccines based on circumsporozoite protein, since the incorporation of polymorphic epitope regions is unlikely to provide heterologous protection if the vaccine also induces non-crossreactive responses.

It was difficult to understand how such a large family of variants, where each one "altruistically" minimises cross-reactivity, may have been selected (Davenport, 1995). The existence of specific aa variation at predicted non-MHC binding residues in the immunodominant Th2R epitope region of CS suggested that it may be under an unusual form of selective pressure. Natural variation in cytotoxic epitope regions of hepatitis B virus and HIV have been shown to behave as altered peptide ligands, providing an immune escape strategy for these viruses (Bertoletti *et al.*, 1994, Klenerman *et al.*, 1994). Malaria has also been shown to utilise APL as an immune evasion strategy via 2 allelic CTL epitopes of the Tc3R region of CS (Gilbert *et al.*, 1998). The study showed that parasites bearing two epitope variants of an HLA-B35 restricted CS epitope may gain a mutual advantage by reciprocal APL antagonism of CTL lytic activity.

I thus investigated whether CD4 T cell variant epitopes within the Th2R region of CS might antagonise one another, and indeed found that selected variants downregulate IFN-γ and proliferative responses by preferential induction of IL-10. Clones capable of high IL-10 secretion, with low or absent secretion of other lymphokines such as IL-2, IL-4, IL-5, or IFN-γ, have been denoted Tr1 (Groux *et al.*, 1997). Here, I show that the ratio of IFN-γ to IL-10 can be changed during both rapid polyclonal responses and when using long term

clones. Therefore, Tr1 may be an inducible phenotype. I induced IL-10 production by an altered peptide ligand, but other stimuli may also lead to a similar rapid conversion. Indeed, changes in lymphokine production could be a useful part of a natural response, switching from early effector cells producing upregulatory cytokines, to a downregulatory phenotype at a later stage.

Interleukin 10 is a pleiotropic lymphokine that can down-regulate MHC class II molecules on APCs, inhibit nitric oxide production, prevent T cell priming and proliferation, and suppress IFN-γ, TNFα, GM-CSF and LT production by T cells (Moore *et al.*, 1993, Le Moine *et al.*, 1999, Akdis and Blaser, 1999, Groux and Powrie, 1999). Thus, IL-10 inducing APL bearing strains may generally benefit the parasite population. The finding that V5 inhibited V6 responses even when presented on separate APCs, lends support to this putative *trans*-effect of IL-10 secretion. The mechanism I observed for V5 might be better described as a suppressive dominant form of "partial agonism", rather than antagonism *per se*, although the term antagonism has been used to describe a variety of related phenomena (Jameson and Bevan, 1995).

The same APL negatively affected both rapid IFN-γ secretion and proliferation in parallel assays in donor 57. This may make this immune evasion mechanism singularly powerful, given our evidence suggesting that these two effector functions may be mediated by different memory T cell subsets (Chapter 3). V7 also induced IL-10 from PBMC, but IL-10 neutralisation did not reverse V7 induced inhibition, suggesting that additional suppressive mechanisms might be operating. Two nonexclusive hypotheses may account for the strong inhibitory effect of APL in polyclonal IFN-γ and proliferative responses. The first hypothesis is that separate T cell populations react to V6 (by IFN-γ production) or V5 (by IL-10 production). When both are induced in the same culture, the IL-10 induced by V5 may non-specifically downregulate V6 IFN-γ responses. However, IFN-γ ELISPOT PPD responses were not inhibited by V5 co-stimulation in parallel assays in our donors in whom V6 inhibition was seen. Thus, IL-10 production alone does not explain the inhibition of V6 responses. An alternative mechanism is that T cells bearing the same TCR,

recognising both V5 and V6, may be susceptible to differential lymphokine regulation by APL antagonism. In support of this hypothesis, differential IFN-γ and IL-10 production to V6 alone, compared to V6 co-presented with V5, could be demonstrated at the clonal level. Once the IFN-γ / IL-10 ratio decreases for a single clone, the IL-10 produced may play a positive feedback role in promoting an IL-10 biased response.

Naturally occurring Th2R specific CD4 T cell responses may therefore change qualitatively in response to co-stimulation by APL-bearing parasites. IFN-γ secreting T cells capable of changing toward IL-10 secretion may not be restricted to the Th2R epitope region in malaria, and may even be a common occurrence. Indeed, a study of malaria infected children and adults in Gabon observed that approximately half of the IL-10 producing CD4 and CD8 T cells co-express IFN-γ (Winkler *et al.*, 1998, Winkler *et al.*, 1999). The authors suggest that this might reflect an indirect feedback mechanism for inhibition of IFN-γ expression. I further propose that such cells may represent transitory populations capable of changing from IFN-γ to IL-10 secretion, and may thus provide a fertile ground for parasite driven immune modulation. Preliminary studies of rapid IL-10 secretion in adult Gambian donors suggested that the majority (10 / 12) have high background levels of IL-10 secretion, although how this phenomenon relates to malaria exposure remains to be determined. Similar cell populations capable of simultaneous IFN-γ and IL-10 production have been described in other diseases such as Lyme disease, and murine viral bronchiolitis (Hussell *et al.*, 1996). Indeed, rapid IFN-γ / IL-10 modulation is likely to play a pivotal role in both infectious and autoimmune disease progression (Mahanty and Nutman, 1995, O'Garra *et al.*, 1997, Trinchieri, 1997, Cobbold and Waldmann, 1998, Hill, 1998, Mason and Powrie, 1998).

High levels of IL-10 present in the liver may hinder T cell priming by hepatocytes (Le Moine *et al.*, 1999). Thus IL-10 induction by agonist / antagonist Th2R epitopes is likely to prevent the priming of IFN-γ producing cells (Moore *et al.*, 1993, Groux *et al.*, 1997) and extend the benefit to the parasite population by generating a population of susceptible hosts (Plebanski *et al.*, 1999). This may provide one more mechanism whereby lifelong

malaria exposed individuals exhibit unusually low levels of T cell reactivity to *P. falciparum* (Doolan *et al.*, 1993, Doolan *et al.*, 1994, Plebanski *et al.*, 1997a). The mechanism I describe in this chapter results in rapid suppression of potentially protective responses. It may also explain, through inefficient priming in an IL-10 rich milieu, how both low levels of reactivity are generated and maintained, and how a family of allelic immunodominant epitopes are selected and maintained.

CHAPTER 5

T CELL RESPONSES TO THROMBOSPONDIN RELATED ADHESIVE PROTEIN (TRAP): COMPARISONS BETWEEN EAST AND WEST AFRICANS

5.1

INTRODUCTION

In the previous chapter I provided several reasons why CS protein may not be the ideal vaccine candidate antigen for induction of protective CD4 T cell responses. It is highly polymorphic in its immunodominant CD4 T cell epitope regions, and cross-strain recognition of polymorphic variants of one such region was infrequently seen in adult Gambians. Moreover, naturally occurring allelic variants of the immunodominant CD4 T cell epitope region, denoted Th2R, acted as altered peptide ligands and switched off protective IFN-γ T cell responses in favour of production of the immunosuppressive Th2 type T cell cytokine IL-10. There is therefore increasing interest in identifying alternative antigens for use in pre-erythrocytic vaccines. Thrombospondin related adhesive protein (TRAP) may be such a candidate antigen.

*Pf*TRAP, also known as sporozoite surface protein 2 (SSP2) (Rogers *et al.*, 1992), is discussed in detail in chapter 1. It is less polymorphic than CS protein (Robson *et al.*, 1990, Robson *et al.*, 1998), although most nucleotide changes result in amino acid changes (Robson *et al.*, 1990). TRAP is required for motility of sporozoites, is released onto the sporozoite surface upon contact with target cells, and is involved in invasion of and entry into hepatocytes. There is evidence in animals and humans that TRAP is a protective antigen. Protective CTL have been demonstrated in murine models of malaria (Khusmith *et al.*, 1991, Khusmith *et al.*, 1994, Schneider *et al.*, 1998,), and several HLA restricted CTL TRAP epitopes have been identified in irradiated sporozoite immunized human volunteers (Wizel *et al.*, 1995b, Wizel *et al.*, 1995a, Doolan *et al.*, 1997), and naturally exposed adult donors (Aidoo *et al.*, 1995, Plebanski *et al.*, 1997a). Potentially protective antibodies to

TRAP have been demonstrated in the murine model (Müller *et al.*, 1993), and naturally exposed humans (Scarselli *et al.*, 1993). Despite increasing evidence for a protective role for $CD4^+$ T cells at the pre-erythrocytic stage of infection, no CD4 T cell epitopes in *Pf*TRAP have been identified.

5.2
STUDY DESIGN

5.2.1
Rationale

This study aimed to map CD4 T cell epitopes within *Pf*TRAP by screening for IFN-γ ELISPOT responses to TRAP derived peptides, using PBMC from naturally exposed Africans. It was also of interest to assess the degree of induction of Th2 type T cell cytokines IL-4 and IL-10 in selected donors, since it is not known if TRAP induces such responses. The degree of cross-recognition of naturally occurring variants of selected TRAP T cell epitopes was assessed by IFN-γ ELISPOT, to see if multiple variants could be recognised by the same donor. Given that CS variant epitope regions were susceptible to APL (Chapter 4), the differential induction of IFN-γ or IL-10 by variants of the same T cell epitope region within TRAP was assessed for in selected donors, as was IL-4 induction by variant peptides in the same donors.

5.2.2
Donors and Study Area

The Gambia

Peripheral blood mononuclear cells (PBMC) from 42 healthy adult male volunteers from Dampha Kunda, Upper River Division, The Gambia (Figure 3.2) were collected and frozen between March and April 1997. To compare reactivity in freshly isolated PBMC, a further 8 healthy adult male and female donors from the village of Brefet, Western Division, The Gambia (Figure 3.2) were tested at the time of our study in December 1997. Thus a total of 50 Gambian donors were recruited to the study. High malaria transmission in The Gambia

occurs between August and November, commencing shortly after the rainy season begins, and thus all samples were taken during the dry season.

Kenya

Sixty five adult Kenyans were recruited between mid June and mid July 1998, which is at the beginning of the high transmission season (June to August) (Mbogo *et al.*, 1995). They were either healthy adult male blood transfusion donors (n = 42, D1-D42), or malaria exposed African employees based at Kilifi district hospital, coastal Kenya (n = 23; 15 male / 8 female) (Figure 5.1). A further 25 male blood transfusion donors (K1-K25) were recruited in September 1999 for the TRAP variant T cell epitope analysis. This represents a period of low malaria transmission in Kilifi. The donors were recruited from the whole of Kilifi district, both North and South of the creek. Malaria transmission intensity varies for different regions of Kilifi, but is generally regarded as low intensity in Northern Kilifi, and high intensity in Southern Kilifi (Gupta *et al.*, 1999).

Malaria naïve donors

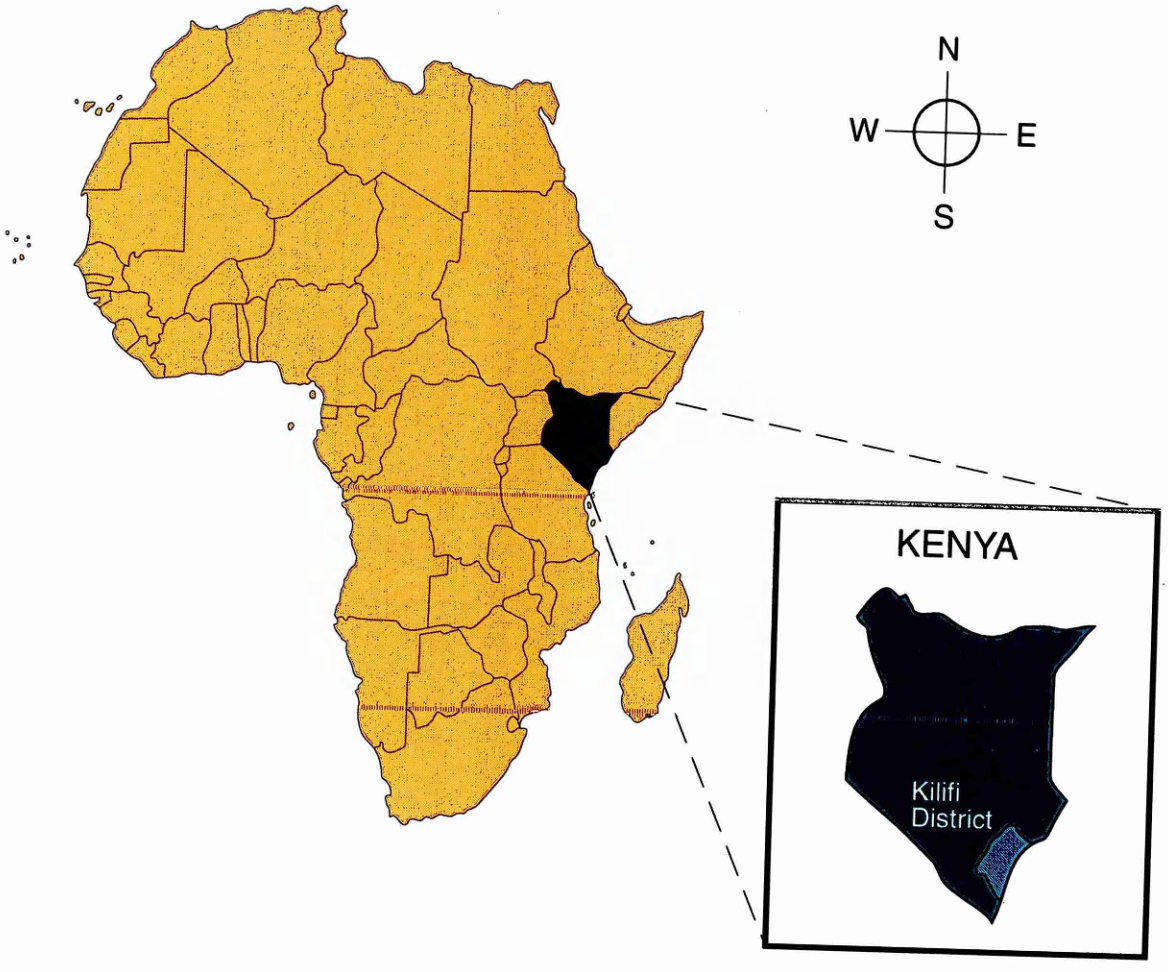
Fifteen malaria naïve adult employees at the John Radcliffe Hospital in Oxford were recruited and tested in the UK under identical conditions to The Gambian and Kenyan samples.

5.2.3

Peptides

A panel of fifty 20mer peptides (tp1-tp50; aa 1-510), overlapping by ten amino acids, derived from *Pf*TRAP clone NF54 were synthesized commercially (Research Genetics) (Figure 5.2, Table 5.1 for aa sequences). Three *Pf*TRAP 20mers covering the carboxy-terminus region (tp51, tp52 and tp53; aa 526-545, 541-560 and 555-574) were synthesized using a standard Fmoc/t-butyl solid-phase Zinsser Analytical synthesizer (Zinsser Analytic) (Table 5.1). Eleven TRAP pools were made by combining the above TRAP peptides (Table 5.2), and were used in selected donors to narrow down the positive response before testing to individual peptides.

Figure 5.1



65 adult Kenyans were recruited from Kilifi District in Kenya. The study area is indicated on the map, and included donors from the Northern district (low to moderate transmission) and the Southern District (high transmission).

Figure 5.2

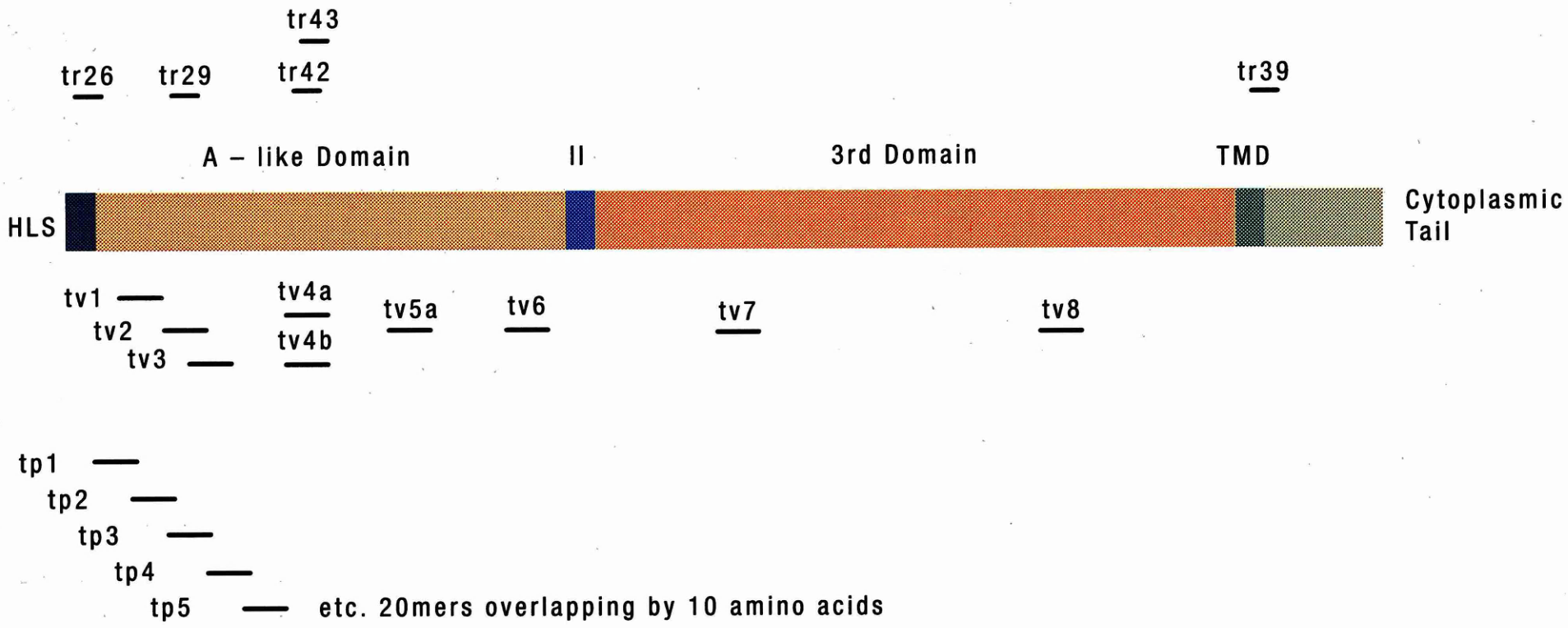


*Diagram of TRAP indicating the positions of the peptides used in the study. The tp peptides were 20mers spanning to residue 510; the tv peptides were those predicted to have a high binding to HLA-DRB1*01or DRB1*1302; the tr peptides were the 5 previously identified CTL epitopes which were tested in selected donors.*

Table 5.1

PEPTIDE	AA	SEQUENCE	CON / VAR
tp1	1-20	MNHLGNVKYLVIVFLIFFDL	Conserved
tp2	11-30	VIVFLIFFDLFLVNGRDVQN	Conserved
tp3	21-40	FLVNGRDVQNNIVDEIKY**R**E	Variant
tp4	31-50	NIVDEIKY**R**EEVCND**E**VD**L**Y	Variant
tp5	41-60	EVCND**E**VD**L**YLLMDCSGSIR	Variant
tp6	51-70	LLMDCSGSIRRHNWV**N**HAVP	Variant
tp7	61-80	RHNWV**N**HAVPLAMKLIQQLN	Variant
tp8	71-90	LAMKLIQQLNLN**D**NAIHLY**A**	Variant
tp9	81-100	LN**D**NAIHLY**A**S**V**FSNNA**R**EI	Variant
tp10	91-110	**S**VFSNNA**R**EIIRLHSDASKN	Variant
tp11	101-120	IRLHSDASKNKEKALIII**K**S	Variant
tp12	111-130	KEKALIII**K**SLL**S**TNLP**Y**G**K**	Variant
tp13	121-140	LL**S**TNLP**Y**G**K**TNL**T**DALL**Q**V	Variant
tp14	131-150	TNL**T**DALL**Q**VRKHLNDRINR	Variant
tp15	141-160	RKHLNDRINRENANQLVVIL	Conserved
tp16	151-170	ENANQLVVILTDGIPDSIQD	Conserved
tp17	161-180	TDGIPDSIQDSLKESR**K**L**S**D	Variant
tp18	171-190	SLKESR**K**L**S**D**R**GVKIAVFGI	Variant
tp19	181-200	**R**GVKIAVFGIGQQGINVAFNR	Variant
tp20	191-210	GQQGINVAFNRFLVGCHPSDG	Conserved
tp21	201-220	FLVGCHPSDGKCNLYADSAW	Conserved
tp22	211-230	KCNLYADSAWENVKNVIGPF	Conserved
tp23	221-240	ENVKNVIGPFMKAVCVEVEK	Conserved
tp24	231-250	MKAVCVEVEKTASCGVWDEW	Conserved
tp25	241-260	TASCGVWDEWSPCSVTCGKG	Conserved
tp26	251-270	SPCSVTCGKGTRSRKREILH	Conserved
tp27	261-280	TRSRKREILHEGCTSE**L**QEQ	Variant
tp28	271-290	EGCTSE**L**QEQCEEERC**L**PK**R**	Variant
tp29	281-300	CEEERC**L**PK**R**EPLDVP**D**EPE	Variant
tp30	291-310	EPLDVP**D**EPEDDQPRPRGDN	Variant
tp31	301-320	DDQPRPRGDN**F**A**V**E**K**P**N**E**N**I	Variant
tp32	311-330	**F**A**V**E**K**P**N**E**N**IIDNNPQEPSP	Variant
tp33	321-340	IDNNPQEPSPNPEEGK**G**EN**P**	Variant
tp34	331-350	NPEEGK**G**EN**P**N**N**GFDLDENPE	Variant
tp35	341-360	**N**GFDLDENPENPPNPPNPPN	Variant
tp36	351-370	NPPNPPNPPNP**P**NP**P**NPPNP	Variant
tp37	361-380	P**P**NP**P**NPPNPDIP**E**Q**E**PNIP	Variant
tp38	371-390	DIP**E**Q**E**PNIPEDSEKEVPSD	Variant
tp39	381-400	EDSEKEVPSDVPKNPEDDRE	Conserved
tp40	391-410	VPKNPE**D**DREEN**F**DI**P**KKPE	Variant
tp41	401-420	ENFDIPKKPENK**H**DNQNNLP	Variant
tp42	411-430	NK**H**DNQNNLPNDKSDR**Y**IPY	Variant
tp43	421-440	NDKSDR**Y**IPYSPL**S**P**K**VLDN	Variant
tp44	431-450	SPL**S**P**K**VLDNERKQSDPQSQ	Variant
tp45	441-460	ERKQSDPQSQDNNGNRHVPN	Conserved
tp46	451-470	DNNGNRHVPNSEDRET**R**PHG	Variant
tp47	461-480	SEDRET**R**PHGRNNENRSYNR	Variant
tp48	471-490	RNNENRSYNRK**H**N**N**TPK**H**PE	Variant
tp49	481-500	K**H**N**N**TPK**H**PEREEHEKPDNN	Variant
tp50	491-510	REEHEKPDNNK**K**K**A**GSDN**K**Y	Variant
tp51	526-545	AGLAYKFVVPGAATP**Y**AGEP	Variant
tp52	540-559	**Y**AGEPAPFDETLGEEDKDLD	Variant
tp53	555-574	EDKDLDEPEQFRLPEENEWN	Conserved

Overlapping TRAP 20mer peptides. Variant residues indicated in bold type.

Table 5.2

Pool Name	Peptides
p1	tp1, tp11, tp21, tp31, tp41
p2	tp2, tp12, tp22, tp32, tp42
p3	tp3, tp13, tp23, tp33, tp43
p4	tp4, p14, tp24, tp34, tp44
p5	tp5, tp15, tp25, tp35, tp45
p6	tp6, tp16, tp26, tp36, tp46
p7	tp7, tp17, tp27, tp37, tp47
p8	tp8, tp18, tp28, tp38, tp48
p9	tp9, tp19, tp29, tp39, tp49
p10	tp10, tp20, tp30, tp40, tp50
p11	tp51, tp52, tp53

TRAP peptide pools used for screening for responses.

The variant TRAP peptides, derived from 10 naturally occurring strains of *Pf*TRAP (Table 5.3), were synthesised commercially (Research Genetics). PPD (Statens Seruminstitut) was utilised as a positive control for the IFN-γ ELISPOT, and RPMI alone as the negative control.

TRAP derived peptides predicted to have a high likelihood of binding to certain HLA class II haplotypes by a computer algorithm programme (Davenport *et al.*, 1995a) were also selected for this study (see Methods). I screened for binding to two different HLA types: HLA-DRB1*1302 which is common in The Gambia (approximately 17% of healthy adults) and is associated with protection against malaria in West Africa (Hill *et al.*, 1991); and HLA-DRB1*01 which is relatively uncommon in The Gambia (approximately 4%), is not associated with protection in Gambians, but is protective in East Africans (A.V.S. Hill, personal communication).

Table 5.3

PEPTIDE	SEQUENCE	STRAIN
tp2	VIVFLIFFDLFLVNGRDVQN	CONSERVED
tp4	NIVDEIKY**R**EEVCND**E**VDL**Y**	NF54/FCR3/K1/DD2/HB3/7901/7G8/3D7/ITO4
tp4x	NIVDEIKY**S**EEVCND**Q**VDL**Y**	T996
tp5	EVCND**E**VDL**Y**LLMDCSGSIR	NF54/FCR3/K1/DD2/HB3/7901/7G8/3D7/ITO4
tp5x	EVCND**Q**VDL**Y**LLMDCSGSIR	T996
tp6	LLMDCSGSIRRHNWV**N**HAVP	7G8/T996/ITO4/FCR3/K1/7901/3D7/ITO4
tp6x	LLMDCSGSIRRHNWV**K**HAVP	DD2/HB3
tp14	TNL**T**DALL**Q**VRKHLNDRINR	NF54/HB3/T996/3D7
tp14x	TNL**S**DALL**Q**VRKHLNDRINR	ITO4/FCR3/K1/DD2/7G8
tp14y	TNL**T**DALL**E**VRKHLNDRINR	7901
tp23	ENVKNVIGPFMKAVCVEVEK	CONSERVED
tp30	EPLDVP**D**EPEDDQPRPRGDN	NF54/T996/3D7
tp30x	EPLDVP**H**EPEDDQPRPRGDN	ITO4/K1/FCR3
tp30y	EPLDVP**Q**EPEDDQPRPRGDN	7901/7G8/DD2/HB3
tp31	DDQPRPRGDN**F A**V**E**KP**NEN**I	NF54
tp31x	DDQPRPRGDN**S S**V**Q**KP**EEN**I	T996/7G8
tp31y	DDQPRPRGDN**F A**V**E**KP**KEN**I	ITO4/3D7/FCR3/7901
tp31z	DDQPRPRGDN**FA**V**E**KP**EEN**I	K1/DD2/HB3
tp37	PPNPPNPPNPDIP**EQE**PNIP	ITO4/3D7/HB3/DD2/FCR3
tp37x	PPNPPNPPNPDIP**EQK**PNIP	T996/NF54
tp37y	PPNPPNPPNPDIP**ERK**PNIP	7901
tp37z	PPNPPNPPNPDIP**QQE**PNIP	7G8/K1
tp38	DIP**EQE**PNIPEDSEKEVPSD	ITO4/3D7/HB3/DD2/FCR3
tp38x	DIP**EQK**PNIPEDSEKEVPSD	T996/NF54
tp38y	DIP**ERK**PNIPEDSEKEVPSD	7901
tp38z	DIP**QQE**PNIPEDSEKEVPSD	7G8/K1
tp40	VPKNPE**D**DREEN**F**DI**P**KKPE	CONSERVED
tp43	NDKSDR**Y**IPYSPL**S**P**K**VLDN	NF54
tp43v	NDKSDR**S**IPYSPL**P**P**K**VLDN	ITO4/K1/FCR3
tp43w	NDKSDR**Y**IPYSPL**P**P**N**VLDN	7G8
tp43x	NDKSDR**N**IPYSPL**P**P**K**VLDN	T996
tp43y	NDKSDR**Y**IPYSPL**A**P**K**VLDN	3D7
tp43z	NDKSDR**Y**IPYSPL**P**P**K**VLDN	7901/HB3/DD2
tp47	SEDRET**R**PHGRNNENRSYNR	CONSERVED
tp51	AGLAYKFVVPGAATP**Y**AGEP	CONSERVED
Pool 4-6a: tp4 + tp5 + tp6		NF54/7G8/3D7/FCR3/K1/7901/ITO4
Pool 4-6b: tp4x + tp5x + tp6		T996
Pool 4-6c: tp4 + tp5 + tp6x		DD2/HB3

Variant peptides selected for the study in Kenyan adults. NB tp40, 47 and 51 are conserved across the 10 strains, but not worldwide. Variant residues are in bold type.

I scanned all known TRAP sequences worldwide using the prediction programme (Davenport *et al.*, 1995a), and 9 peptides (tv1-tv8) which had a high predicted binding affinity to either of the two haplotypes were selected, including two polymorphic regions (tv4 and tv5) (Table 5.4A). These were synthesized using a standard Fmoc/t-butyl solid-phase Zinsser Analytical synthesizer. These were made into 3 pools for preliminary screening studies in certain donors (Table 5.4B).

Table 5.4

A.

PEPTIDE	AA	SEQUENCE	DRB1*1302 BINDING	DRB1*01 BINDING	ORIGIN
tv1	13-32	VFLIFFDLFLVNGRDVQNNI	1.16	1.27	Conserved
tv2	34-53	DEIKYSEEVCNDQVDLYLLM	1.05	1.27	Thailand
tv3	44-58	NDQVDLYLLMDCSGS	0.94	1.21	Thailand
tv4a	89-108	YANIFSNNAKEIIRLHSDAS	1.26	1.04	Indochina/Braz/Honduras
tv4b	89-108	YASVFSNNAKEIIRLHSDAS	1.27	1.04	Africa
tv4c	89-108	YVNVFSNNAKEIIRLHSDAS	1.06	1.16	W.Africa/Thailand
tv5a	124-38	TNLPYGRTNLTDALL	0.92	1.16	Africa/Thai/Honduras
tv5b	124-38	TNLPYGRTNLSDALL	0.92	1.14	Thai/Indochina/Brazil
tv6	164-78	IPDSIQDSLKESRKL	1.23	1.12	Thai/Indoc/Afr/Hond/Braz
tv7	251-65	SPCSVTCGKGTRSRK	1.29	1.18	Conserved
tv8	431-50	SPLPPKVLDNERKQSDPQSQ	1.28	1.17	Thai/Indoch/Ugand/Hond

B.

POOL NAME	PEPTIDES
v1	tv1, tv4a, tv5a, tv8
v2	tv2, tv4b, tv5b, tv7
v3	tv3, tv4c, tv6

*TRAP tv peptides selected according to high predicted binding to 2 common HLA haplotypes, DRB1*01 and DRB1*1302. Predicted binding values are given for each peptide (>1.2 is considered to be a high predictive value).*

5.2.4

Statistical Methods

Proportions of donors responding in different studies were compared used standard chi squared analysis, having first established that the background levels were comparable. Where the numbers in each group for comparison were very small, Fisher's value for 2-tailed analysis was calculated for the χ^2 p-value. The precursor frequency values (SFU / 10^6 PBMC) were compared using t-tests to assess for differences between groups.

5.3

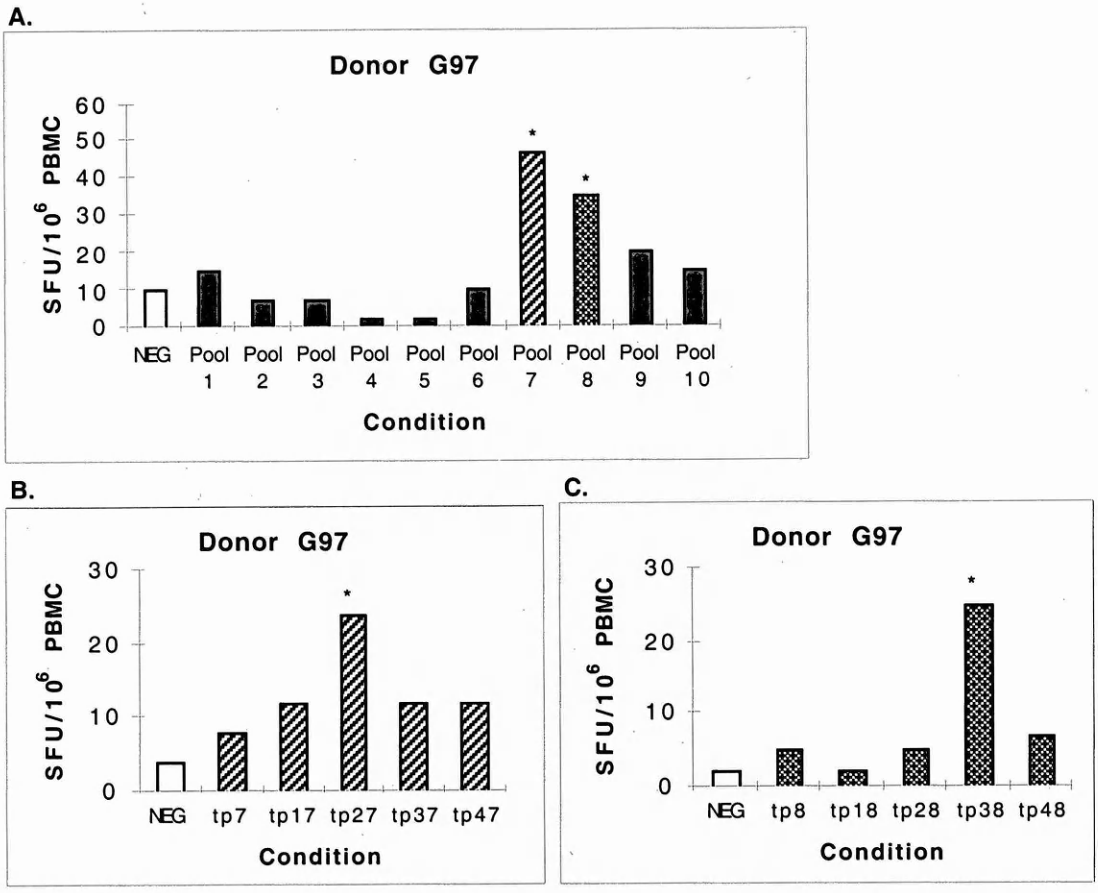
RESULTS

5.3.1

IFN-γ Secreting T Cells From Semi-immune Gambian Adults Recognise Multiple Epitopes Along the Length of *Pf*TRAP

Forty one of 50 individuals recruited to the Gambian study were tested using frozen peripheral blood mononuclear cells (PBMC) for early IFN-γ ELISPOT responses to the 50 overlapping 20mers (tp1-tp50) spanning amino acid residues 1-510 of *Pf*TRAP (Table 5.1). They were screened first for responses to 10 pools of 5 peptides per pool (Table 5.2), and where a positive response was seen to a pool (Figure 5.3A) they were screened to the individual peptides within that pool (Figures 5.3B and C). A positive response was assigned if the peptide test well response was regarded as significantly above background ($P \leq 0.05$) according to the statistical table discussed in methods. Thirty two percent (13/41) of individuals screened had T cell responses to a defined tp TRAP peptide (Table 5.5A). Over half of those who gave a positive IFN-γ ELISPOT responded to one peptide only, and the maximum number of tp epitopes to which an individual responded was five (donor G73). The positive responses ranged from 13 to 89 spot forming units (SFU, see Methods) per 10^6 PBMC with an arithmetic mean of 28.2 ±1.2 SE (Table 5.5A). The responses were repeated at least twice for each donor (pools and individual peptides), and were consistent on re-testing up to 4 times per donor.

Figure 5.3



*Mapping for IFN-γ ELISPOT responses to TRAP peptides involved first screening for positive responses to pools of TRAP peptides (A). Where a positive response to pools was observed, the constituent peptides within that pool were tested (B and C). Donor G97 thus responded to peptide tp27 (contained in pool 7) and tp38 (contained in pool 8). * represents a significant positive response above background ($p < 0.05$).*

I therefore detected between 13 and 89 circulating T cells per million in peripheral blood capable of producing IFN-γ in response to individual *Pf*TRAP epitopes. The fact that the numbers were low suggested that only low levels of circulating T cells recognise TRAP antigen in immune individuals. The levels were comparable to the precursor frequency levels reported for CD8 T cells to TRAP using chromium release assays in naturally exposed donors of 16 to 86 per million PBMC (Plebanski *et al.*, 1997a). Low level T cell reactivity is a well recognised feature of natural immunity to malaria, and is discussed in

the introduction (Chapter 1). Eleven of the 41 donors above, and a further 6 donors not screened above, were tested for reactivity to 3 peptides covering the 48 terminal amino acids of the carboxy-terminus of TRAP (tp51-tp53, aa 526-574) (Table 5.1). None of these seventeen individuals gave a positive response, however six of them gave positive responses to other TRAP peptides in this study. Seventy percent of donors gave a positive PPD response (64 ±9 SE SFU / 10^6 PBMC).

I thus identified 21 novel T cell epitopes denoted tp within *Pf*TRAP which lead to IFN-γ production in Gambian adults, by screening 47 of the 50 individuals recruited to this study (Tables 5.5). Seventeen of these epitopes (81%) fall in polymorphic regions of TRAP, which is not surprising since TRAP has numerous point mutations along its length (Robson *et al.*, 1990, Robson *et al.*, 1998). Many of the variant epitopes identified contain polymorphisms which are exclusive to malaria endemic regions of the world other than Africa, such as Southeast Asia and Central America. Taking this into account, the number of conserved West African tp epitopes identified in this study was nine (Table 5.5). The four that are conserved worldwide (tp22, tp23, tp24, tp25) span aa residues 211-260, and are clustered in a highly conserved region towards the end of the 'A-like domain' (aa 48-241) and incorporating most of the Region II sulphatide binding domain (aa 246-267) (Figure 5.2). The remaining five which are conserved in West Africa (tp6, tp14, tp39, tp40, tp41) are distributed along the length of TRAP.

5.3.2

Double the Reactivity to TRAP in East Africans Compared to West Africans

A similar study to The Gambian one described above was performed using PBMC from healthy adult Kenyans from Kilifi district, coastal Kenya (Figure 5.1). It was not known if differences in reactivity would exist between the two African populations. This is clearly an important consideration in terms of vaccines designed to be effective in different malaria endemic regions of the world. Thus, 65 healthy adult Kenyans were recruited, and their PBMC were tested for reactivity to the same panel of overlapping 20mer TRAP peptides (tp1-tp53) used in The Gambia (Table 5.1).

Table 5.5

A. IFN- γ ELISPOT Responses in Adult Gambians

EPITOPE	FREQ	DONORS	SFU/10^6	P-VALUE	WORLDWIDE	WEST AFRICA
tp4	2%	G58	15	0.05	Variant	Variant
tp5	2%	G110	20	0.05	Variant	Variant
tp6	2%	G99	25	0.05	Variant	Conserved
tp10	2%	G73	13	0.05	Variant	Variant
tp11	5%	G73/131	40/17	0.005/0.05	Variant	Variant
tp13	2%	G73	23	0.05	Variant	Variant
tp14	5%	G73/85	20/13	0.05/0.05	Variant	Conserved
tp22	2%	G55	20	0.05	Conserved	Conserved
tp23	2%	G110	20	0.05	Conserved	Conserved
tp24	2%	G58	30	0.05	Conserved	Conserved
tp25	2%	G132	21	0.05	Conserved	Conserved
tp27	2%	G97	36	0.05	Variant	Variant
tp33	2%	G102	22	0.05	Variant	Variant
tp38	2%	G101	23	0.01	Variant	Variant
tp39	2%	G132	24	0.05	Variant	Conserved
tp40	2%	G95	21	0.05	Variant	Conserved
tp41	2%	G95	48	0.001	Variant	Conserved
tp43	2%	G73	33	0.005	Variant	Variant
tp47	2%	G60	53	0.005	Variant	Variant
tp48	2%	G46	46	0.001	Variant	Variant
tp50	7%	G85/60/102	32/60/30	0.05/0.001/0.05	Variant	Variant

B. IFN- γ ELISPOT Responses in Adult Gambians

EPITOPE	FREQ	DONORS	SFU/10^6	P-VALUE	WORLDWIDE	WEST AFRICA
tv1	6%	G73	25	0.05	Conserved	Conserved
tv4a	12%	G73/96	48/20	0.001/0.05	Variant	Variant
tv5a	12%	G85/35	13/23	0.05/0.01	Variant	Variant

C. IL-4 ELISPOT Responses in Adult Gambians

EPITOPE	FREQ	DONORS	SFU/10^6	P-VALUE	WORLDWIDE	WEST AFRICA
tv2	7%	G35	28	0.05	Variant	Variant
tv4b	7%	G35	33	0.01	Variant	Variant
tp11	17%	G110	13	0.05	Variant	Variant

Summary of all positive ELISPOT responses in the study of 50 adult Gambians. The magnitude of each positive response is given (SFU/10^6 PBMC), and the p-value for the SFU (spot forming units) value above the background response.

IFN-γ responses were assessed using a standard overnight ELISPOT assay as before. The first 22 donors were tested using the same pools of peptides tested in The Gambia (Table 5.2), and thereafter donors were tested immediately to the individual tp peptides without first screening for responses to pooled peptides.

Fifty five percent (12/22) of the Kenyan donors screened for responses to the pooled peptides responded to one or more pools (data not shown). This is almost double the reactivity rate of 32% seen in the Gambian study. This high level of reactivity was confirmed for the remaining 43 Kenyan adults who were screened to individual peptides. Sixty three percent of these (27/43) gave a positive response to at least one of the TRAP peptides tp1-tp50 (Table 5.6). Therefore, there was a significantly higher response rate to TRAP in Kenyans compared to Gambians (χ^2 = 8.13, p = 0.004) (Table 5.7A). The increased TRAP reactivity observed in Kenyan compared to Gambian adults was no longer significant if only the TRAP pool responders were compared (χ^2 = 3.12, p = 0.08), which is probably a reflection of the smaller sample size for the Kenyans. Reactivity to 9 Th2R and 8 Th3R CD4 T cell epitope variants of CS were assessed in parallel to TRAP responses in 64 Kenyan donors. I found an overall response rate of 32.8% (21/64) to these 17 epitopes in the Kenyan adults, compared to 46.8% (22/47) reactivity in a similar study using PBMC from Gambian adults from Dampha Kunda and Brefet (not shown). Thus, there was no significant difference in the T cell reactivity to CS protein between adults from the 2 countries (χ^2 = 2.24, p = 0.13) (Table 5.7A). The number of donors capable of giving a positive TRAP peptide T cell IFN-γ response was thus greater in the Kenyans compared to Gambians. The magnitude of positive T cell responses (specific responder cells / 10^6 PBMC) were comparable between the Kenyan donors (arithmetic mean 33.6 SFU ±2.1 SE / 10^6 PBMC), and Gambian donors (28.2 ±1.2 SE / 10^6 PBMC) (p = 0.25) (Table 5.7B). Eighty six percent (56/65) of the Kenyan donors gave a positive PPD response, which is significantly higher than the 70% (33/47) of Gambians who responded (χ^2 = 4.25, p = 0.04) (Table 5.7A). The PPD precursor frequencies (SFU/10^6 PBMC) were also significantly higher in Kenyans compared to the Gambians (p = 0.03) (Table 5.7B).

Table 5.6

IFN-γ ELISPOT Responses in Adult Kenyans

EPITOPE	DONORS	FREQ	SFU/10^6 PBMC	P-VALUES
tp1	D11	2%	30	0.05
tp2	D11/23/34,OK	9%	33,43,23,95	0.05,0.01,0.05,0.01
tp3	D16,OK	5%	18,108	0.05,0.01
tp4	D12/34/37/39,OK	12%	23,20,18,80,43	0.05,0.05,0.05,0.01,0.05
tp5	D19/23/24/30	9%	28,15,55,43	0.05,0.05,0.05,0.01
tp6	D16/21/27/34/36	12%	20,18,18,25,18	0.05,0.05,0.05,0.01,0.05
tp7	D11/23,OK	7%	23,18,55	0.05,0.05,0.05
tp8	D23/27	5%	35,15	0.01,0.05
tp9	D35	2%	20	0.05
tp12	D11/28	5%	23,40	0.05,0.05
tp13	D23	2%	18	0.05
tp14	D11/14/16/17	9%	30,18,35,20	0.05,0.05,0.01,0.05
tp15	D11/13	5%	25,28	0.01
tp16	D11/13	5%	25,23	0.05,0.05
tp17	D11	2%	22	0.05
tp18	D28/40	5%	48,23	0.01,0.05
tp19	D13/42,OK	7%	30,30,90	0.01,0.01,0.01
tp20	D40,OK	5%	20,48	0.05,0.05
tp21	DM	2%	18	0.05
tp22	D23/27	5%	23,28	0.05,0.01
tp23	D11/14/19	7%	28,30,28	0.05,0.05,0.05
tp24	D14	2%	23	0.05
tp25	D11/27	5%	28,18	0.05,0.05
tp26	D11/27	5%	35,18	0.05,0.05
tp27	MN,D13,OK	7%	15,20,55	0.05,0.05,0.05
tp28	D13/34,OK	7%	20,25,68	0.05,0.01,0.01
tp29	D37,OK	5%	28,45	0.01,0.05
tp30	D21/27/34	7%	20,20,30	0.05,0.05,0.01
tp31	D23/34/39,OK	9%	18,23,30,63	0.05,0.05,0.05,0.01
tp32	OK	2%	148	0.001
tp34	D40	2%	18	0.05
tp35	D11	2%	23	0.05
tp36	D11/16	5%	28,20	0.05,0.05
tp37	D23/34/40,OK	9%	20,33,23,63	0.05,0.01,0.05,0.01
tp38	D11/23/34,OK	9%	25,23,20,60	0.05,0.05,0.05,0.05
tp39	D21/34	5%	18,20	0.05,0.05
tp40	D14/34/40,OK	9%	18,30,18,75	0.05,0.01,0.05,0.01
tp42	MN,D11	5%	15,23	0.05,0.05
tp43	BM,TK	5%	25,23	0.05,0.05
tp44	OK	2%	83	0.01
tp45	PN	2%	40	0.01
tp47	D24/25,JN,OK	9%	55,20,60,43	0.05,0.05,0.001,0.05
tp48	D34,OK	5%	18,80	0.05,0.01
tp49	D25,OK	5%	20,85	0.05,0.01
tp50	D11	2%	30	0.05
tp51	D12/14/15/19/25/40	14%	28,28,23,28,30,20	0.01,0.05,0.05,0.05,0.05,0.05
tp52	TK,D12/34	7%	23,25,20	0.05,0.01,0.05
tp53	D12,OK	5%	20,88	0.05,0.01

Table 5.7

A. Number of Responders

	Kenya	The Gambia	p-value
PPD	56 / 65 86%	33 / 47 70%	**0.04***
TRAP (50 peptides)	27 / 43 63%	13 / 41 32%	**0.004***
CS (18 peptides)	21 / 64 33%	22 / 47 47%	0.13

B. Magnitude of Response

	Kenya	The Gambia	p-value
PPD	122 +/-7	64 +/-9	**0.03***
TRAP Peptides	34 +/-4	37 +/-6	0.25
CS Peptides	42 +/-4	ND	ND

A significantly higher proportion of adult Kenyan donors responded to TRAP derived peptides, compared to adult Gambians. The PPD response rate was also higher, but was of borderline significance. The magnitude of the response (specific responder cells / 10^6 PBMC) was higher in Kenyans for PPD responses (backgrounds were comparable), but the TRAP peptide specific values were comparable between the 2 groups.

5.3.3

Peptides Tested According to High Predicted Binding to HLA-DRB1*01 or DRB1*1302

Seventeen of the 50 donors recruited to The Gambian study were tested for IFN-γ production against nine of the eleven tv peptides (tv4c and tv5b were not tested in this study) (Table 5.4A). These peptides were selected according to a high predicted binding to either HLA DRB1*1302, a common African haplotype (17% Gambians, 14% Kenyans) which is thought to be protective in West Africa (Hill *et al.*, 1991), or HLA DRB1*01 which is infrequent in The Gambian population (4% Gambians, 10% Kenyans), and associated with protection in East Africa (A.V.S. Hill, personal communication). Four Gambian adult donors (18%) gave a positive response to a tv peptide, suggesting that this method of trying to identify new epitopes according to HLA binding motifs is no more fruitful than conventional methods using overlapping peptides. Of the 3 IFN-γ stimulating epitopes identified in this way (Table 5.5B) one is conserved (tv1), and the remaining two (tv4a and tv5a) fall within polymorphic regions of TRAP. Two (tv1 and tv5a) are high predicted binders to HLA DRB1*01, which is not associated with protection in The Gambia, and one (tv4a) to the common allele HLA DRB1*1302, which is protective. Interestingly tv4a has not been isolated in Africa, unlike the other 2 variants tv4b and 4c which are African strains (Table 5.4A).

Forty three Kenyan adults were tested for responses to all 11 tv peptides, and eleven (26%) of them gave a positive response to at least one (Table 5.8). This is slightly higher than the number of Gambian responders, although 2 more peptides were used in this study. A comparison of the response rate in Kenyans (23.3%, 10/43) to the same 9 tv peptides tested in The Gambians (22.2%, 4/18) showed almost identical reactivity rates to peptides selected in this manner ($\chi^2 = 0.01$, $p = 0.93$). The commonest T cell response in Kenyans was to peptide tv7 (5/43, 12% of donors), followed by peptide tv8 (9% of donors). Neither of these are predicted high binders to the protective HLA DRB1*01 allele in East Africa. Epitope tv7 is conserved, thus it is a potentially useful vaccine candidate, and tv8 has been identified as a strain present in East Africa (Table 5.4A).

Table 5.8

EPITOPE	DONORS	FREQ	SFU/10^6 PBMC	P-VALUES
tv1	D11/21	5%	23,23	0.05,0.05
tv2	D12/23/39	7%	33,20,30	0.01,0.05,0.05
tv3	D12/34	5%	18,20	0.05,0.05
tv4a	D12	2%	23	0.05
tv4b		0%		
tv4c	D16,OK	5%	23,45	0.05,0.05
tv5a	D11	2%	23	0.05
tv5b	D11	2%	23	0.05
tv6	D12/19	5%	18,38	0.05,0.05
tv7	D11/13/23,OK	9%	17,25,18,80	0.05,0.05,0.05,0.01
tv8	D11/19/21/34,OK	12%	23,33,20,33,83	0.05,0.05,0.05,0.01,0.01

*IFN-γ ELISPOT responses in Kenyan adults to the 11 tv TRAP peptides selected according to high predicted binding to 2 common HLA haplotypes, DRB1*01 and DRB1*1302 (see Table 5.4).*

There seems to be no relationship between epitope recognition for TRAP and predicted HLA binding to a locally protective class II haplotype. Four of the tv peptides have not been isolated from infected Africans to date (tv2, tv3, tv4a, tv5b) and are regarded as exclusive to other malaria endemic regions of the world (Table 5.4A). Nevertheless, all were recognised by Kenyan adults suggesting that either these donors have generated cross-reactive responses to variants that are present locally, or they are present but have not been isolated. The TRAP variant strains present in infected West Africans are well established (Robson *et al.*, 1998), and preliminary data for Kenya suggests that the African strains common in West Africa predominate (K. Robson, personal communication). Thus these responses are probably truly cross-reactive.

5.3.4

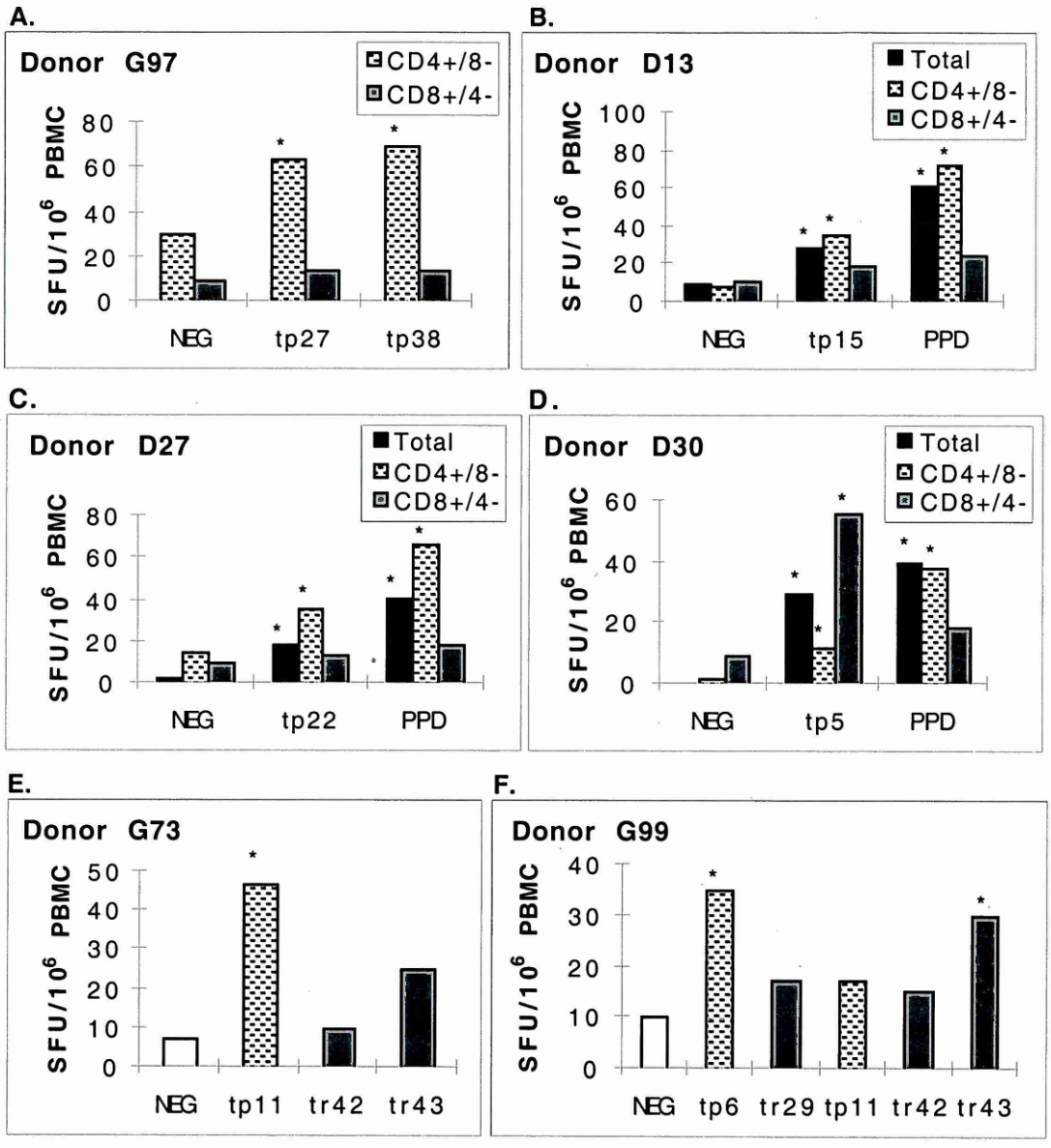
The Majority of 20mer TRAP Epitopes Elicit $CD4^+$ T Cell Responses

CD4 and CD8 T cell depletion studies were performed to establish the phenotype of the responding T cells where possible (see Methods). Depletion studies of Gambian PBMC samples confirmed that IFN-γ ELISPOT responses to tp11, tp27, and tp38 were CD4 T

cell mediated (Figures 5.4A, Table 5.9). Depletion of Kenyan adult PBMC confirmed that responses to a further 13 of the *Pf*TRAP tp epitopes and one tv epitope elicited $CD4^+$ T cell IFN-γ ELISPOT responses (Figures 5.4B and C, representative examples of 13 experiments, Table 5.9). The PPD responses behaved as predicted and disappeared following CD4 T cell depletion, but were unaffected by CD8 T cell depletion (Figures 5.4 B, C and D). Interestingly, two of the tp TRAP peptides (tp5 and tp6) elicited either CD4 or CD8 responses, even in the same donor in one case (D30) (Figure 5.4D). Since both tp5 (aa 41-60) and tp6 (aa 51-70) contain the CD8 9mer epitope tr29 (aa 51-59), then presumably these donors were able to process this epitope from the 20mer during an overnight ELISPOT assay. Dual CD4/CD8 T cell epitope regions have similarly been described for CS protein (Doolan *et al.*, 1991).

Four of the 5 CTL epitopes used in our studies (tr26, tr29, tr42, tr43) are contained in tp 20mer peptides (see Table 5.10), and in certain cases I found reactivity to either the CD4 epitope or the CD8 epitope contained within it, but not to both. For example the TRAP peptide tp11 (aa 101-120) contains two known overlapping CD8 T cell epitopes tr42 (aa 107-115) and tr43 (aa 109-117). In the Gambian study donor G73 responded to tp11, but failed to respond to tr42 or tr43 (Figure 5.4E). Conversely, donor G99 responded to the 9mer CTL epitope tr43 (aa 109-117), a response likely to be CD8 mediated, but failed to respond to tr42 or tp11 (Figure 5.4F). Donor G99 also responded to tp6 (aa 51-70), but not to the constituent CTL epitope tr29 (aa 51-59) (Figure 5.4F). These findings were confirmed in the Kenyan study where more than 10 donors responded to a TRAP 20mer tp epitope without responding to the CTL epitope contained within it (not shown). Therefore, a positive response to the 20mer peptide need not be due to presentation of the known CTL epitope contained within that 20mer peptide, although these CTL epitopes can be processed.

Figure 5.4



CD4 and CD8 depletion studies in Gambians (G73, G97 and G99) and Kenyans (D13, D27 and D30) demonstrated that most tp 20mer TRAP epitope responses were CD4 T cell epitopes in nature (A, B and C). Two peptides, tp5 and tp6, were found to elicit both CD4 and CD8 responses, even in the same donor in one case (D). A TRAP 20mer was recognised without a concomitant response to the CTL epitope contained within it (E and F) and vice versa (F).

Table 5.9

EPITOPE	CD4 or CD8	DONORS KENYAN	GAMBIAN
tp4-6 pool	CD4	K7	
tp4-6 pool	CD4 & CD8	K23	
tp5	CD4 & CD8	D30	
tp6	CD4 & CD8	D16	
tp9	CD4	D10	
tp11	CD4		G131
tp14	CD4	D16	
tp15	CD4	D13	
tp19	CD4	D13	
tp22	CD4	D27	
tp23	CD4	D11,K13	
tv7	CD4	D11	
tp26	CD4	D11	
tp27	CD4		G97
tp31	CD4	K6	
tp32	CD4	D4	
tp37-38 pool	CD4	K13	
tp38	CD4		G97
tp43	CD4	K10	
tp47	CD4	JN	
tp50	CD4	D11	
tp51	CD4	D8	

Summary of results of all CD4 and CD8 depletion studies. Most of the 20mer peptides tested were CD4 T cell epitopes.

Table 5.10

PEPTIDE	AA	CONTAINED IN	SEQUENCE	HLA
tr26	3-11	tp1	HLGNVKYLV	A2
tr29	51-59	tp5, tp6	LLMDCSGSI	A2
tr39	515-23	No	GIAGGLALL	A2
tr42	107-15	tp11	ASKNKEKAL	B8
tr43	109-17	tp11	KNKEKALII	B8

Summary of the 5 CTL epitopes tested in selected donors, including the HLA restriction. Four of them are contained within the tp 20mer peptides used in the study.

5.3.5

T Cell Repertoire Differences Between East and West Africans

Interestingly, the repertoire of T cell responses in Kenyans compared to Gambians was quite different. The commonest TRAP T cell epitope response in Kenyans was to peptide tp51 (aa 501-520) to which 14% of Kenyan adults responded (Table 5.6), yet none of the 17 Gambians tested gave a positive response (Table 5.5A). Conversely, tp50 (aa 511-530) was the commonest peptide recognised by Gambian donors (7%), but was only positive for one of the 65 Kenyan donors tested (2%). Figure 5.5 highlights these differences in T cell repertoire between the 2 populations. One striking feature of this figure is that of the very few TRAP peptides which the Kenyans failed to respond to (5 in total), responses were seen to all but one of them in Gambian adults. This is in the presence of a considerably lower reactivity rate in the Gambian population, and strongly suggests that repertoire differences do exist between the 2 populations.

The data for the tv peptides, which were selected according to high predicted HLA binding capacity, further supports our hypothesis that the T cell repertoire of responses differ between the 2 populations. The two most commonly recognised peptides in the Gambian study were tv4a and tv5a, which were positive in 12% of donors tested, whereas only 2% (1/43) of Kenyan adults recognised the same peptides in a comparable study. Conversely, no Gambian donors recognise tv7 or tv8 which were so commonly positive among Kenyan adults (12% and 9% respectively).

There are many reasons why such reactivity differences might exist between the 2 populations. These include differences in HLA type between East and West Africans, differences in parasite strains present in these 2 regions, and differing levels of parasite exposure. I have established that most of these TRAP 20mers elicit CD4 T cell responses (Section 5.3.4) and thus the class II HLA types of 29 Kenyan responders, and 14 of the Gambian responders, were established by phototyping (see Methods). This demonstrated that the HLA types were indeed quite different between the 2 populations (Table 5.11).

Figure 5.5

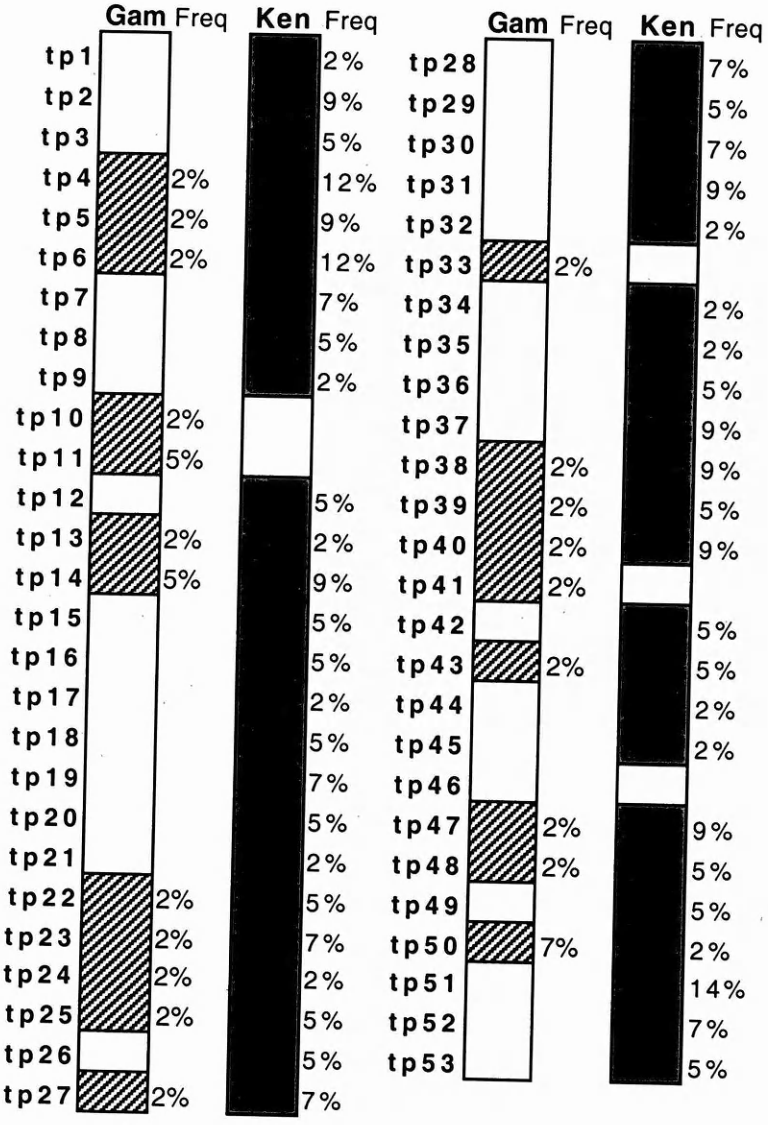


Figure to illustrate the differences in repertoire of TRAP peptide responses between East (65 adult Kenyans) and West (47 adult Gambians) Africans. Where ≥1 positive response was observed in the study the bar is filled in. The Gambians responded to less peptides than the Kenyans. One striking feature is that the Gambian donors responded to 4 of the 5 peptides which the Kenyans failed to respond to (tp10, tp11, tp33, tp41 and tp46) strongly suggesting repertoire differences.

Table 5.11

A. Kenyan Adults

DONOR	DRB1	DRB1	DRB5	DRB3	DRB4	DQB1	DQB1	tp PEPTIDE RESPONSES
BM	7	13		52	53	2	5	tp43/tv7
DM	11	?		52		4		tp21
JN	18			52		4		tp47
MN	11	13		52		5	7	tp27,42
OK	17			52		5	2	tp2-4,7,19,20,27-9,31-2,37-8,40,44,47-9,53/tv4c,7,8
PN	13			52		6	7	tp45
TK	12		51			5	6	tp43,52
D11	10	11		52		5		tp1,2,7,12,14-17,23,25-6,35-6,38,42,50/tv1,5a,5b,7,8
D12	7	13		52	53	7		tp4,51,52,53/tv2,3,4a,6
D13	1	11		52		5	7	tp15,16,19,27,28/tv7
D14	17			52		2	5	tp14,23,24,40,51
D15	11	12		52		5	7	tp51
D16	11	15	51	52		5	6	tp3,6,14,36/tv4c
D17	11	?	51	52				tp14
D19	11			52		5	7	tp5,23,51/tv6,8
D21	1	13		52	53	2	5	tp6,30,39/tv1,8
D23	7	13		52	53	2	7	tp2,5,7,8,13,22,31,37,38/tv2,7
D24	11	17		52		5		tp5,47
D25	11	12		52		5		tp47,49,51
D27	18			52		5		tp6,8,22,25,26,30
D28	11			52		6		tp12,18
D30	11	13		52		5	6	tp5
D34	7	12		52	53	2	5	tp2,4,6,28,30,31,37-40,48,52/tv3
D35	13	17		52		2	6	tp9
D36	10	11		52		5		tp6
D37	13	17		52	?53	2		tp4,29
D39	18			52		4	5	tp4,31/tv2
D40	13			52		5		tp18,20,34,37,40,51
D42	13			52		6		tp19

B. Gambian Adults

Donor	DRB1	DRB1	DRB5	DRB3	DRB4	DQB1	DQB1	tp Peptide Responses
G35	ND	ND	ND	ND	ND	ND	ND	tv5a
G46	ND	ND	ND	ND	ND	ND	ND	tp48
G55	11	1304		52		7		tp22
G58	1302	1304/12		52		6	7	tp4,24
G60	16	1301	51	52		5	6	tp47,50
G73	10	1302		52		5	6	tp10,11,13,14,43,tv1,tv4a
G85	18	1304		52		4		tp14,50,tv5a
G95	8	1303		52		7		tp40,41
G96	11	1304		52		7		tv4a
G97	9	11		52	53	7	2	tp27
G99	9	1302		52	53	6	2	tp6
G101	15	11	51	52		6	7	tp38
G102	8	9			53	7	2	tp33,50
G110	1302	1321		52		6	7	tp5,23
G131	18	1304		52		4	7	tp11
G132	4	10			53	5	8	tp25,39

Kenyan and Gambian peptide responders were HLA class II typed. No specific HLA type could be assigned to a particular epitope response from this data.

It was not possible from this data to assign a particular HLA class II type to particular TRAP T cell epitope responses since no common class II types emerged for individual epitopes. This in itself is very interesting since it may mean that the TRAP epitopes are recognised through multiple HLA types, rather than just one. This would certainly be advantageous in terms of vaccine design.

Parasite strains have been extensively typed in West Africa (Robson *et al.*, 1988, Robson *et al.*, 1990, Robson, 1993, Robson *et al.*, 1996, Robson *et al.*, 1998), and preliminary data for certain Kenyan donors that were blood film positive in this study suggested that similar strains were present in Kenya in comparable frequencies, although some new mutations do exist (K. Robson, personal communication). Differences in rates of parasite exposure, and differences in strains are discussed in conclusions.

5.3.6

Malaria Naïve Individuals Do Not Respond to TRAP 20mer Peptides

To confirm that the responses were TRAP specific, fifteen malaria naïve donors were tested in Oxford for IFN-γ ELISPOT responses to the 10 pools of non-overlapping 20mer TRAP peptides tp1-tp50 (aa 1-510) (Table 5.3) using freshly isolated PBMC. The conditions used were identical to those used in The Gambia and Kenya, and assays were performed with the same peptides and batch tested serum and media. Seventy three percent (11/15) of the malaria naïve donors gave a positive response to PPD, and none of the 15 naïve donors gave a positive response to the TRAP pools containing peptides tp1-tp50 (not shown). The lack of responses observed in naïve adults is highly significant when compared to Gambian adults ($\chi^2 = 6.19$, $p = 0.01$) and Kenyan adults ($\chi^2 = 17.56$, $p = 0.00003$). Naïve donors were not assessed for responses to the tv peptides.

5.3.7

Reactivity to Polymorphic Variants of TRAP T Cell Epitopes in Kenyans

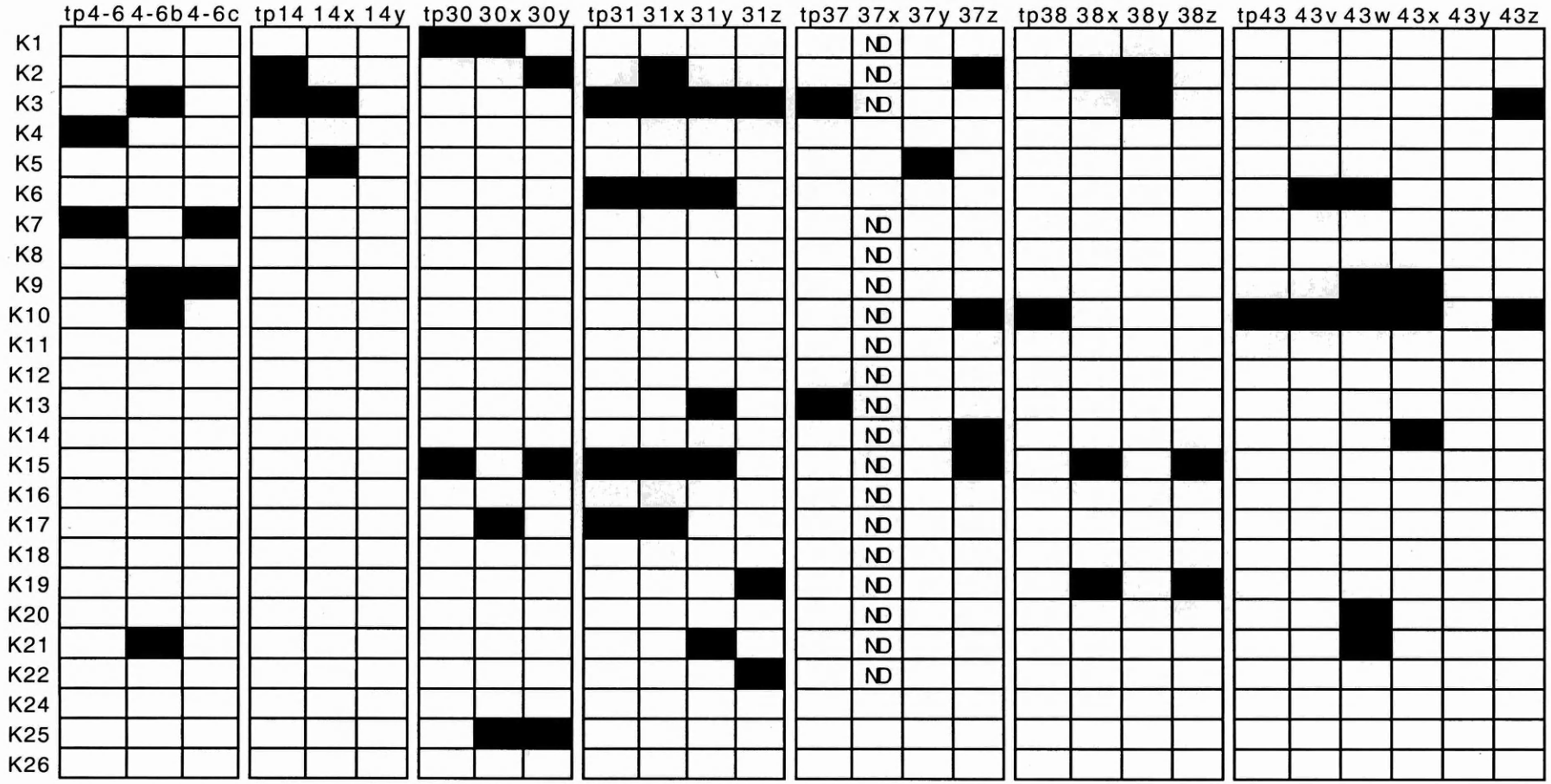
One of the reasons for the interest in TRAP as a malaria vaccine candidate is that it is less polymorphic than CS, although up to 5 naturally occurring variants have been identified for

some regions of the molecule. Throughout this chapter I have assessed for T cell responses to peptides derived from a single clone of *Pf*TRAP, called NF54. It was not known how much reactivity would be found to multiple variants of single polymorphic T cell epitope regions of TRAP. In order to assess this I selected nine of the most immunogenic polymorphic TRAP tp epitopes from the Kenyan study, and synthesised the polymorphic variants from 10 naturally occurring strains (Table 5.3). These strains have been isolated and cloned from parasites from all over the world including East (7901) and West Africa (3D7, 7G8, ITO4, NF54), Latin America (HB3), and South East Asia (DD2, K1, T996) (Robson *et al.*, 1998).

Twenty five Kenyan adults were tested by IFN-γ ELISPOT to these variant peptides from PfTRAP. Twenty seven peptide conditions were tested per donor, and 72% (18/25) of donors responded to one or more condition (Table 5.12). Forty percent (10/25) of the donors responded to the NF54 strain variant, thus assessing for responses to the 10 variant strains almost doubled the response rate to a single strain. Forty four percent (11/25) of the donors tested showed a degree of cross-recognition of different variant epitopes within variant T cell epitope regions. The majority of cross-recognition occurred to 2 variant T cell epitopes only, although 4 donors responded to 3 or 4 variants of a particular T cell epitope. Three of these latter multiple strain responses were to epitope tp31, although 5 of the 9 donors who gave a tp31 region response did so to one variant only. The tp31 epitope region contains the proposed cell recognition sequence, RGD, which is likely to be of functional significance.

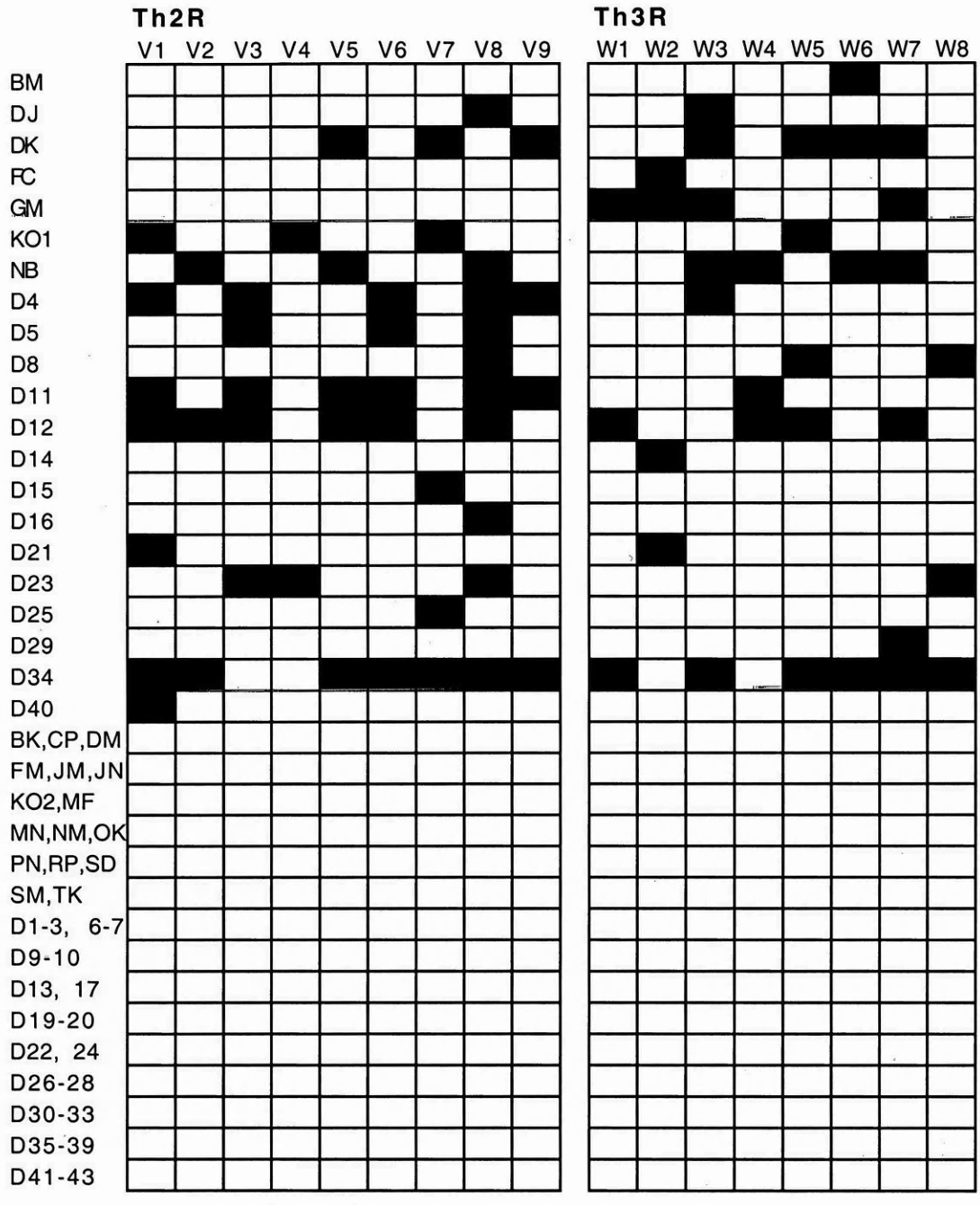
Thirty nine percent (16/41) of positive responses to any particular epitope region exhibited a degree of cross-recognition, although there was only one instance in which a donor responded to all the variants of a particular epitope (donor K3 to all 4 tp31 variants) (Table 5.12). Studies of cross-recognition of the highly variable Th2R and Th3R CD4 T cell epitope variants of CS by IFN-γ ELISPOT in Kenyan adults showed that 56.3% (9/16) of the Th2R responders and 40% (6/15) of the Th3R responders showed a degree of cross-recognition (Table 5.13).

Table 5.12



26 adult Kenyan donors were tested by IFN-γ ELISPOT assay to the variant peptides derived from 10 naturally occurring strains (see Table 5.3). A positive response is indicated by a filled in square. The degree of cross-recognition of naturally occurring variant epitopes was limited.

Table 5.13



65 Kenyan adult donors were tested by IFN-γ ELISPOT assay for responses to the 9 Th2R epitope variants and 8 Th3R epitope variants of CS protein. Positive responses are indicated with filled in squares. Limited cross-recognition of variants was seen.

Comparing the degree of cross-recognition to the TRAP epitope variant regions, with that seen to the immunodominant Th2R and Th3R CS epitope regions, suggested that there is no difference in cross-recognition of responses between these 2 important antigens (χ^2 = 0.63, p = 0.43). This comparison is not entirely valid since CS is considerably more polymorphic than TRAP, and the study assessed responses to 2 polymorphic epitope regions of CS, and 7 variant epitope regions of TRAP. However, both studies addressed reactivity to a similar number of naturally occurring strains (9 for CS protein, 10 for TRAP), and the results suggest similar levels of cross-recognition.

5.3.8

Th2 Type T Cell Responses in Gambian and Kenyan Adults

Preliminary studies screening for Th2 type responses in selected Gambian donors showed minimal Th2 induction. Thus, adult Gambians were screened for IL4 (n = 6) and IL10 (n = 8) responses to the 10 pools of *Pf*TRAP 20mer peptides (clone NF54) spanning residues 1-510 (Table 5.2). Where a positive response was seen, the individual peptides within the pools were tested. PHA was used as the positive control in all assays. Only one of the six individuals tested for IL4 production gave a positive response (Table 5.5C), and none of the eight tested gave an IL10 response. Additionally, selected Gambian adults were screened for responses to the 3 peptides, tp51-53, spanning the carboxy-terminus of TRAP, and to the 9 tv peptides. No donors responded to tp51-53 by IL4 (n = 13) or IL10 (n = 2) production. One of the fifteen donors tested for IL4 production responded to two tv peptides (tv2 and tv4b) (Table 5.5C). Neither of the two individuals screened for IL10 responses were positive to the tv peptides, or indeed any other TRAP peptides.

A more comprehensive study of Th2 type T cell reactivity to TRAP was performed using PBMC from Kenyan blood transfusion donors (1998 cohort). Twenty three donors were assessed in total, 22 by IFN-γ, 21 by IL-4, and 8 by IL-10 ELISPOT assays (Table 5.14). Fifty nine percent (13/22) of donors responded to at least one pool by IFN-γ production, and 33% (7/21) produced IL-4 (Table 5.14). The higher level of IFN-γ reactivity compared to IL-4 reactivity failed to reach significance (χ^2 = 2.87, p = 0.09).

Table 5.14

DONOR	IFN-γ	IL-4	IL-10
TK	p2,p11	v1	p11
GM	v1	nil	nil
KO1	p5,p6	p11,v1,v2,v3	p1,p5
MM	ND	p8	p1,p5,v3
DJ	p2,p3,v3	nil	nil
JN	p7	p1,p11,v1	p5
D43	nil	ND	p1,p6,v1
D22	nil	ND	nil
D10	p9	nil	ND
MF	nil	nil	ND
BM	p3,v2	nil	ND
D34	p1-2,p4,p6-11,v1,v3	nil	ND
DM	p1	p9	ND
D9	nil	nil	ND
D17	p4	nil	ND
D19	p3,p5,p11,v1,v3	nil	ND
D21	p6,p9,p10,v1	nil	ND
D32	nil	nil	ND
D37	p4,p9	p2,p7	ND
NB	p4	nil	ND
RP	nil	p7	ND
D31	nil	nil	ND
D20	nil	nil	ND
	13 / 23 (57%)	7 / 21(33%)	5 / 8 (63%)

Selected Kenyan adult donors were screened in parallel IFN-γ, IL-4 and IL-10 ELISPOT assays for responses to pools of TRAP peptides. ND = not determined.

Where IFN-γ and IL-4 responses were detected in the same donor, they were to different peptide pools. For example, donor D37 had an IFN-γ response to pools 4 and 9, and an IL-4 response to pools 2 and 7 (Table 5.14). Sixty three percent (5/8) of the donors tested for gave an IL-10 response (Table 5.14). Two of the 5 responders produced IFN-γ and IL-10 to the same peptide pools (donors TK, KO1), although I do not know to which individual peptides. I thus detected similar levels of IFN-γ and IL-10 reactivity ($\chi^2 = 1.39$, $p = 0.24$), but higher IL-10 reactivity (63%) compared to IL-4 reactivity (33%), although the

difference was not significant ($\chi^2 = 2.03$, $p = 0.22$), probably because of the small sample size.

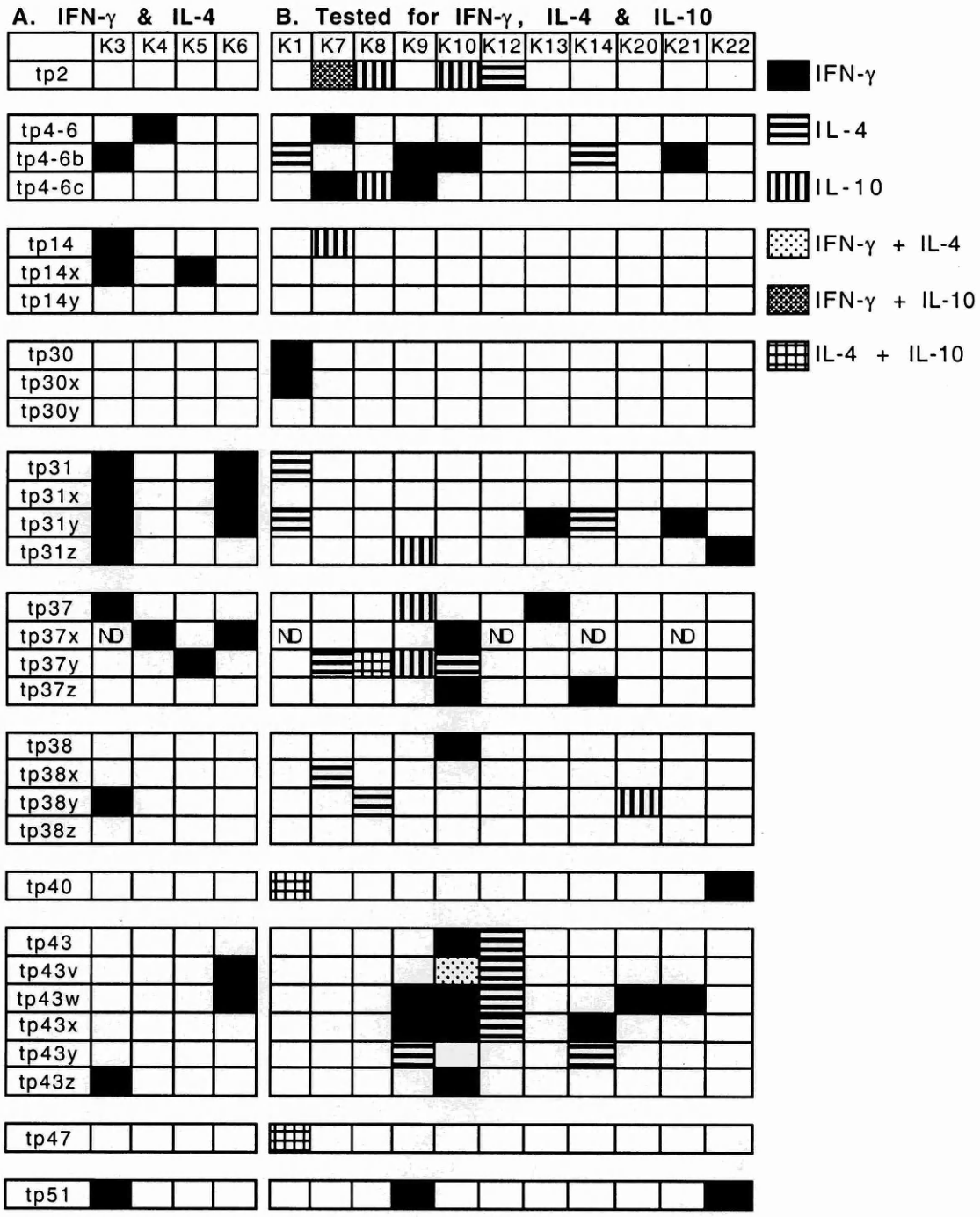
The West African study of Th2 T cell reactivity was less comprehensive but certain differences between East and West Africans were apparent. The same number of donors were tested in Kenya and The Gambia (n = 8) for IL-10 production to the 10 TRAP pools, and while none of The Gambian donors responded to the TRAP pools, half the Kenyan donors (4/8) gave an IL-10 response ($\chi^2 = 5.33$, $p = 0.02$), although the significant difference was lost on adjusting for small numbers (2-tailed Fisher's value $p = 0.08$). Seventeen percent (1/6) of the Gambian donors gave an IL-4 response to TRAP pools, and 29% (6/21) of Kenyans to the same pools, analysis of which suggested no significant difference in IL-4 reactivity rates between the 2 countries ($\chi^2 = 0.34$, $p = 1.0$).

5.3.9

Th1 / Th2 T Cell Responses to Variant TRAP T Cell Epitopes

I speculated that individual variant TRAP peptides may selectively induce either IFN-γ or a Th2 (IL-4 or IL-10) type T cell responses, as observed by ELISPOT for CS protein epitope specific T cell responses (Chapter 4). Eleven of the 25 donors assayed for IFN-γ T cell responses to the variant TRAP peptides (Table 5.3) were therefore tested for IL-10 and IL-4 ELISPOT T cell responses in parallel to IFN-γ. A further 4 donors were tested for IFN-γ and IL-4 only. Fifty five percent (6/11) of donors tested for IL-10 ELISPOT reactivity, and 47% (7/15) of donors tested for IL-4 ELISPOT reactivity, gave positive T cell responses to one or more TRAP conditions (Table 5.15). There was no significant difference in the proportion of donors who produced IL-4 or IL-10 to this panel of TRAP T cell variant epitopes ($\chi^2 = 0.16$, $p = 0.69$). The total number of IL-4 responses (n = 20) observed in this variant analysis was greater than the number of IL-10 responses (n = 12). This is in contrast to the greater IL-10 reactivity (63%), compared to IL-4 reactivity (33%), observed to peptides derived from one strain of TRAP (NF54) only (Section 5.3.8), although the difference did not reach significance ($p = 0.22$).

Table 5.15



Kenyan adult blood transfusion donors were tested for responses to the TRAP variant peptides (Table 5.3) by IFN-γ and IL-4 (n = 4 donors) or IFN-γ, IL-4 and IL-10 (n = 11 donors) ex-vivo ELISPOT assays. Positive responses are indicated by filled squares according to the key on the right hand side.

It may be that the NF54 strain of TRAP preferentially induced IL-10 T cell responses, whereas other strains induce IL-4. There were more IL-10 responses (n = 7) to the NF54 strain, than IL-4 responses (n = 5), but the donor numbers were too small to assess for significance. Another possible reason for the high levels of IL-10 induction in the single strain study was that this study was performed during the high malaria transmission season (June/July 1988), whereas the variant study was performed during a low transmission period (September 1999). High levels of IL-10 induction by malaria epitopes is a recognised phenomenon during episodes of malaria transmission (Winkler *et al.*, 1998, Winkler *et al.*, 1999).

Two donors (K8 and K12) gave entirely Th2 biased responses to TRAP variants, and 3 of the 11 donors for whom all 3 cytokines were tested were Th1 biased (donors K13, K21, K22) (Table 5.15). For three of the donors IFN-γ was produced to certain variants, and IL-4 to another variant of the same epitope (donors K9, K10, K14). For example donor K10 produced IFN-γ to tp37x and 37z, and IL-4 to tp37y. Similarly, donor K14 produced IFN-γ to tp43x and IL-4 to tp43y. There were no cases of IL-10 production to one variant and IFN-γ to another. Thus, I could find no TRAP variant CD4 T cell regions that might behave as altered peptide ligands in a similar manner to that described for CS in chapter 4. The conserved peptide tp2 induced an IFN-γ and IL-10 response in the same donor (K7). Another donor produced IL-4 and IL-10 to the conserved epitope tp47 (K1). This dual induction of different cytokines by the same conserved CD4 T cell epitopes illustrates the complexity of malaria T cell immunity.

5.4

DISCUSSION

Thrombospondin related adhesive protein (TRAP) is thought to play a role in protection at the pre-erythrocytic stage of malaria infection, and might be a useful component of malaria vaccines. In this chapter I examined CD4 T cell reactivity to TRAP in detail, since no CD4 T cell epitopes had been identified for the antigen. I have identified 52 novel 20mer epitopes (21 in exposed Gambians, 48 in exposed Kenyans), denoted tp, spanning the length of the antigen, which can induce rapid IFN-γ production in naturally exposed donors. A further 10 novel IFN-γ inducing epitopes (3 in Gambians, 10 in Kenyans), denoted tv, were identified in these 2 studies, having been selected on the basis of a high predicted binding to certain HLA class II haplotypes. Thus, from 64 peptides I have identified 62 novel epitopes, 16 (26%) of which are conserved worldwide (Table 5.1). The screening for multiple peptide responses in one simple assay thus facilitates epitope mapping for malaria, and may also be utilised for other infectious diseases. Sixteen of the tp epitopes, and one of the tv responses, were shown to be CD4 T cell mediated. Two of the tp responses (tp5 and tp6) were dual CD4 and CD8 T cell epitopes, which is consistent with the fact that they both contain a previously identified HLA A2 restricted CTL epitope called tr29 (Aidoo *et al.*, 1995).

It is interesting that the epitopes were spread along the length of the molecule, unlike CS where regions of immunodominance arise in certain polymorphic areas of the antigen (Good *et al*, 1988d). This suggests that multiple regions of the antigen can be processed and presented to T cells *in vivo*. This is quite unusual, since generally only certain regions of an antigen are immunogenic. This data suggested that TRAP may be under less immune selection pressure than CS protein, which is further supported by the fact that it is less polymorphic than CS protein. Furthermore, this data supports the incorporation of the entire TRAP antigen into a pre-erythrocytic malaria vaccine, since the antigen contains so many T cell epitope regions.

Adult Kenyans showed significantly increased T cell reactivity to TRAP compared to Gambians, but comparable reactivity to two immunodominant CD4 epitope regions of CS (Th2R and Th3R). The high reactivity levels observed in naturally exposed Kenyans suggested that a vaccine bearing one or two strains of TRAP might be sufficient to induce immune responses in most Kenyan donors, although this may not be the case for Gambians The T cell repertoire of IFN-γ responses to TRAP derived peptides in these 2 populations was quite different, and this is an important consideration in the design of vaccines for use in naturally exposed donors. The possible reasons for differences in reactivity rates and repertoire are numerous, and include technical differences in the assays, or real differences. The assays were all performed by myself or under my supervision, using the same methods, and the same peptides and batch tested serum. This therefore minimises the possibility of technical differences, but cannot rule it out. T cell repertoire differences with decreased reactivity in Kenyans and increased reactivity in Gambians was observed for IFN-γ ELISPOT responses to another malaria antigen, MSP-1, in parallel studies (Lee *et al.*, 2000). This further suggested that the differences observed in this study were not technical in nature.

There are many non technical reasons for differences in reactivity between the Gambian and Kenyan adults. These include different HLA types of the 2 populations, different parasite exposure, and different strains and seasonality. Class II HLA typing confirmed considerable disparity between the 2 populations (Table 5.11), and typing of many of the responders failed to show common HLA types for donors giving the same TRAP epitope responses. This suggested that the TRAP epitopes are recognised through more than one HLA type, which may in part explain why so many responses were seen across the whole antigen. This further supports TRAP as an excellent malaria vaccine candidate, since disparity in HLA distributions in different malaria endemic regions of the world is a major obstacle in malaria vaccine design. It remains to be seen whether high levels of T cell reactivity to TRAP occur in other malaria endemic regions of the world.

In The Gambia the entomologic inoculation rate (EIR) is low frequency with an individual receiving an average of 5 infective bites / year (Greenwood *et al.*, 1987, Gupta *et al.*, 1994). In Kilifi, Kenya the parasite prevalence is ~50%, and the EIR ranges from 0-60 infective bites per year depending on where on the coast the individual lives (Mbogo *et al.*, 1995) (low to moderate transmission in North Kilifi, high transmission in South Kilifi). The seasonality is also quite different between the 2 countries. There is one long wet season in The Gambia between August and November when the majority of malaria episodes occur. In Kilifi, transmission occurs most of the year, with peak periods occurring with the rains from June to August and December to January (Mbogo *et al.*, 1995). Thus, the reactivity differences observed in this study might be explained on the grounds of the differences in parasite exposure. The Gambian samples were taken during the low transmission period, and thus IFN-γ ELISPOT T cell responses to TRAP might be expected to be low given that the ELISPOT is thought to reflect recent memory events (Chapter 3). The sampling in Kenya took place during a high transmission period when ELISPOT T cell responses might be expected to be higher. These broad conclusions are complicated by the fact that malaria induced immunosuppression of T cell responses is also well recognised (Ho *et al.*, 1986, Theander *et al*, 1986, Riley *et al.*, 1988a, Hviid *et al.*, 1991b), although in this study it is the more highly exposed Kenyans that exhibit higher reactivity.

An analysis of responses to polymorphic T cell epitope variants of TRAP, derived from 10 naturally occurring strains, showed that for approximately 35% of positive responses there was some cross-recognition with variants of the same epitope. This was comparable to the degree of cross-recognition of T cell variants within 2 immunodominant CS T cell epitope regions. In only one instance did a donor respond to all the variants tested for a particular TRAP T cell epitope (K3 to the 4 tp31 variants tp31, 31x, 31y, 31z). There are several publications detailing the TRAP variant strains present in infected West Africans (Robson *et al.*, 1990, Robson *et al.*, 1998), but only limited data for East African Ugandans (Robson *et al.*, 1998), and no published data for Kenyans. Preliminary typing data for Kenyan donors in this study showed that the strains were similar to those present in The Gambia, but certain differences in frequencies and some new polymorphisms were

observed (K. Robson, personal communication). Interestingly, over 50% of Kenyan donors had mixed infections with more than one parasite strain, which should increase the likelihood of a donor recognising more than one T cell epitope variant at a time. The cross-recognition of variant T cell epitopes by individual donors in this study was probably mediated by different T cell clones, that are each capable of recognising different epitopes within the same region, rather than truly cross-reactive T cell clones which recognise multiple variants of a T cell epitope through the same TCR. However, certain of the variants tested have never been identified in Africans eg the tp43x RNI polymorphism (aa 426-428) (Table 5.3) appears to be exclusively South East Asian and was not found in the Kenyan samples (K. Robson, personal communication). The IFN-γ ELISPOT T cell responses seen for 3 Kenyan donors (K9, 10, 14) to tp43x (Table 5.12) might therefore be truly cross-reactive with T cells which recognise another variant T cell epitope for the same region to which the donors had been exposed. The other possibility is that the strains are present in Kenya, but have not been identified to date, or that positive responses are due to cross-reactivity with another immunogen to which the T cells have been primed (Zevering *et al.*, 1992).

I found double the number of IL-10 compared to IL-4 reactivity in Kenyans (63% vs 33%) to the NF54 strain of TRAP, although this did not reach significance probably because of the small sample size. There was also increased IL-10 reactivity to TRAP in Kenyans compared to Gambians which again failed to reach significance on adjusting for small sample size. One possible explanation for the high IL-10 reactivity in the Kenyan study was that it was performed during the malaria season (June/July), whereas the Gambian study was performed during the dry season when malaria transmission rates were low. The preferential induction of IL-10 is one immunological mechanism by which the parasite might suppress the host immune response during peak transmission periods, and may in part be responsible for malaria induced immunosuppression of T cell responses. Indeed, a study of malaria infected children in Gabon observed many IL-10 producing CD4 and CD8 T cells (Winkler *et al.*, 1998, Winkler *et al.*, 1999). When the TRAP peptides from 10 variant strains were tested for Th2 reactivity in adult Kenyans, the IL-10 and IL-4

response rate was comparable (47% of donors gave IL-4 and 55% IL-10 responses). This study was performed after the peak malaria transmission period in Kilifi, which may explain the reduced level of IL-10 induction. In certain donors variant TRAP peptides from the same T cell epitope region were found to induce either IFN-γ or IL-4. For no epitope region was there a differential induction of IFN-γ and IL-10 in the same donor by variant peptides, and thus no evidence that TRAP polymorphic variants employ the same APL immune evasion strategy described for CS variants in chapter 4. However, the high level of IL-10 reactivity induced by TRAP in Kenyans during the malaria transmission season suggested that preferential induction of this immunosuppressive cytokine might play a role in the well recognised immunosuppression of malaria.

In summary, this study demonstrated that TRAP represents a significant target for protective IFN-γ T cell responses in naturally exposed Gambians and Kenyans, which may be susceptible to boosting by vaccination. A number of conserved epitopes have been identified in the 2 populations, and these may represent particularly useful components of a future malaria vaccine. High levels of IL-10 were induced in those donors tested by ELISPOT during the peak malaria transmission season. Differences in T cell reactivity to TRAP were observed between East and West Africans in terms of the absolute number of responders, although precursor frequency levels were comparable. Studies aimed at understanding differences in T cell reactivity to malaria vaccine candidate antigens in different malaria endemic populations are essential if the goal of developing an effective malaria vaccine effective in these different populations is to be achieved.

CHAPTER 6

TRAP T CELL RESPONSES BY IFN-γ ELISPOT ASSAY: ADULTS COMPARED TO CHILDREN AND ANALYSIS FOR CORRELATES OF PROTECTION

6.1

INTRODUCTION

In the previous chapter I demonstrated differences in T cell reactivity to TRAP between East African (Kenyan) and West African (Gambian) adults. The Kenyans had a significantly greater rate of T cell reactivity to TRAP, but not to CS protein. The repertoire of responses to a panel of overlapping peptides was also different between the 2 populations, although the numbers of specific responder cells (per 10^6 PBMC) were comparable for both TRAP- and CS-specific T cell responses. These apparent population differences might be real, or may be due to technical differences, and these possibilities were explored in the discussion.

The many non-technical variables that complicate a comparison between 2 populations include HLA differences, differences in parasite exposure and strains, and different malaria seasonality. Differences in reactivity between East and West Africa might suggest that the increased reactivity observed in Kenyans was a consequence of the increased exposure to malaria in these individuals compared to Gambians. If this is the case then the fact that reactivity to CS protein was comparable between the 2 populations might reflect negative regulatory factors affecting reactivity to CS protein, but not TRAP. Indeed, I have demonstrated CS protein variant epitope antagonism at the CD4 effector T cell level.

Children are the main target population for malaria vaccine programmes, and it is thus important to understand the nature of their T cell immunity in response to natural exposure, and the type of responses that are to be boosted by vaccination. I therefore undertook to assess TRAP reactivity in children, and to compare reactivity with adults in

order to further understand the nature of development of immunity to TRAP. No studies have directly compared T cell responses to pre-erythrocytic antigens between adults and children. Any differences between subgroups could not be attributed to technical discrepancies, HLA variation, or different parasite strain prevalence, thus ruling these variables out.

Throughout this thesis I have utilised the IFN-γ ELISPOT assay to measure T cell responses to malaria derived peptides. I have suggested that the IFN-γ ELISPOT is a more clinically relevant assay than proliferation, since the Th1 type cytokine IFN-γ is thought to be protective at the pre-erythrocytic stage of infection. Indeed, there is evidence in animals that IFN-γ ELISPOT responses correlate with protection (Plebanski *et al.*, 1998). In humans, the candidate vaccine RTS,S has been shown by IFN-γ ELISPOT to induce CD4 T cell responses to CS protein, and these are thought to be relevant in protection (Lalvani *et al.*, 1999). The ELISPOT is a rapid and simple technique for use in field studies, and can be used to screen for multiple peptide responses at a time.

Despite the possibility that IFN-γ ELISPOT responses might be protective, no study has assessed for a protective association between IFN-γ ELISPOT responses and the subsequent development of malaria in naturally exposed donors. Since I observed such high levels of reactivity to TRAP in Kenyan adults, I proposed to investigate whether IFN-γ ELISPOT responses to TRAP correlated with protection. If protective IFN-γ ELISPOT responses were found, then this assay might provide a simple method of assessing both the levels of protective immunity in the population, and monitoring T cell responses during malaria vaccine trials. The assay could also be used for assessing immunity to other infectious diseases where IFN-γ is thought to play a protective role.

6.2

STUDY DESIGN

6.2.1

Rationale

6.2.1.1

Adults vs Children

Protective immunity at the pre-erythrocytic stage of malaria infection is probably the cumulative effect of responses to hundreds or even thousands of pre-erythrocytic antigens. Development of pre-erythrocytic immunity from childhood to adulthood is poorly understood, and there is a notable paucity of studies comparing T cell responses throughout different age groups. A comprehensive comparison of children and adults might increase our understanding of the development of pre-erythrocytic immunity, and the nature of protection enjoyed by lifelong exposed adults. It is not known if young children have IFN-γ ELISPOT responses to T cell epitopes, or at what age such responses might first be seen. It was also unknown whether the repertoire of T cell responses would vary between age groups, or whether the entire population would have a comparable T cell repertoire given their similarity in exposure rates and HLA type. To address these questions a cross-sectional analysis of IFN-γ ELISPOT responses to selected T cell epitopes from TRAP was performed in a cohort of donors ranging from infants to old age.

6.2.1.2

Analysis for Protection

The ELISPOT is a relatively new tool for use in field studies, and no large scale assessment of ELISPOT responses have looked for protection against the subsequent development of malaria. There is good evidence supporting a protective role for TRAP in animal studies, and some evidence for a protective role in humans (reviewed in Chapter 1). I therefore aimed to assess whether IFN-γ ELISPOT responses to selected TRAP T cell epitopes were protective. Donors tested by IFN-γ ELISPOT in a cross-sectional bleed were subsequently followed up prospectively in the field, and assessed for the development of

malaria. Analysis for protective IFN-γ ELISPOT T cell responses to TRAP was then performed.

6.2.1.3

One Year Repeat ELISPOT Assays

I proposed in chapter 3 that the *ex-vivo* ELISPOT detects circulating effector T cells (T_{EM}). If this is correct one would predict that responses will change frequently over time according to recent exposure. No study has assessed for changes in IFN-γ ELISPOT T cell responses over time in malaria exposed donors, and in fact there is little data regarding such changes with time for any disease. Results from a malaria vaccine trial suggest that CS-specific IFN-γ ELISPOT T cell responses decline after 1 month in malaria naïve donors (Lalvani *et al.*, 1999). A selected number of donors in this study were therefore re-tested in identical IFN-γ ELISPOT assays after 1 year to see whether responses were maintained, or whether they changed after 1 year of natural exposure.

6.2.2

Study Site and Volunteers

Two hundred and seventeen donors aged between 1 month and 81 years (57% females, 43% males) were recruited from Ngerenya district in Northern Kilifi (Figure 6.1). The people were predominantly Giriama ethnicity, and were mainly farmers of maize and cassava for home consumption, and cashew and coconut cash crops. The cross-sectional bleed took place at Kilifi District Hospital (Figure 6.1) between 31st August and 11th September 1998. This represents a period of lower transmission following the peak transmission intensity period from June to August (Mbogo *et al.*, 1995). Ngerenya district is an area of moderate malaria transmission with an entomologic inoculation rate (EIR) of 10 (range 3-15) infectious bites per person / year (C. Mbogo, personal communication)

Figure 6.1

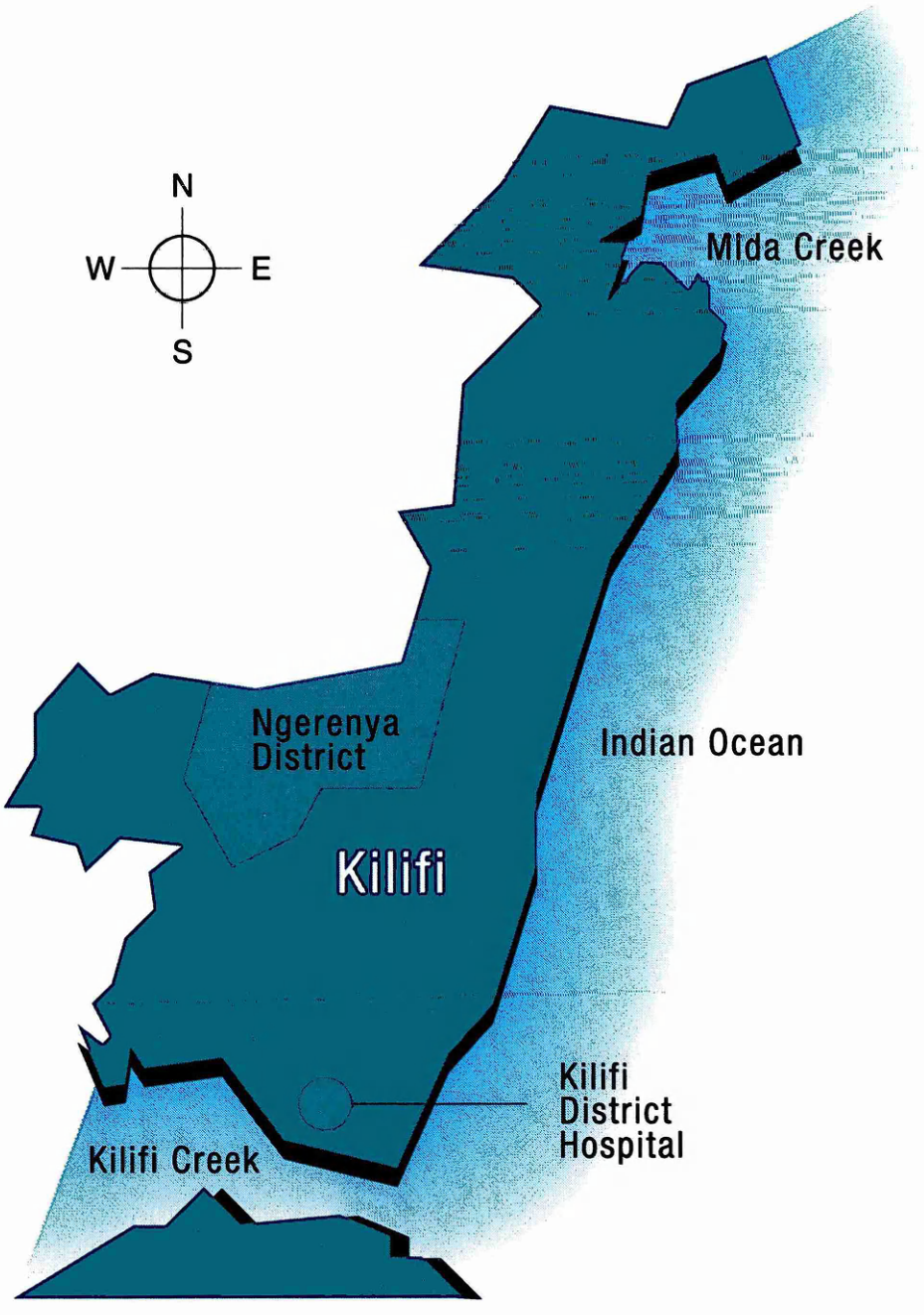


Figure indicating the Ngerenya district study area in Northern Kilifi. 217 donors were recruited from households in Ngerenya, and followed up weekly in the field. The cross-sectional bleed took place at Kilifi District Hospital (indicated).

6.2.3

Peptides

Fourteen tp TRAP peptides were selected for the cross-sectional analysis, and were chosen according to a number of criteria: all those with a frequency of ≥9% in the pilot study of Kenyan adults (Chapter 5, Table 5.6); and those of particular interest such as highly conserved (tp23), highly polymorphic (tp43), or containing a domain of proposed functional significance (tp30) (Table 6.1, 6.2). Peptides tp4, tp5 and tp6 were pooled and tested as one condition, as were peptides tp30 with tp31; and tp37 with tp38. This gave 10 *Pf*TRAP conditions for testing in the donors (Table 6.1). Fifty one percent of the adults in the pilot study responded to one or more of these 10 TRAP conditions. Seven of the 10 TRAP conditions were known to be CD4 T cell epitopes, and one (tp4-6) elicited CD4 or CD8 T cell responses in depletion studies. Two were conserved sequences worldwide (tp2 and tp23), and 6 conserved in Africa (tp2, tp14, tp23, tp40, tp51) (Table 6.1).

Table 6.1

CONDITON	AA	CD4 / CD8	WORLDWIDE	AFRICA	RESPONSE IN GAMBIANS
tp2	11-30	ND	conserved	conserved	No
tp4-6	31-70	CD4 & CD8	variant	variant	Yes
tp14	131-150	CD4	variant	conserved	Yes
tp23	221-240	CD4	conserved	conserved	Yes
tp30-1	291-320	CD4	variant	variant	No
tp37-8	361-390	CD4	variant	variant	Yes
tp40	391-410	ND	variant	conserved	Yes
tp43	421-440	CD4	variant	variant	Yes
tp47	461-480	CD4	variant	variant	Yes
tp51	526-545	CD4	variant	conserved	No

The 14 TRAP peptides selected for testing in the 217 donors were made up into 10 conditions, 3 of which were peptide pools.

Table 6.2

EPITOPE	FREQ	NOTES
tp2	9%	Conserved, mostly signal sequence, predicted β-sheet (aa1-20/28-30)
tp4	12%	S-S aa43 to 55, β-sheet (aa31-33/48-50)
tp5	9%	A-like domain (begins aa48), S-S aa 43 to 55, β-sheet (aa48-53), CTL epitope tr29 (aa51-59)
tp6	12%	In A-like domain, S-S aa55, β-sheet (aa51-53), CTL epitope tr29 (aa51-59, LLMDCSGSI)
tp14	9%	α-helix (aa136-143)
tp23	7%	Highly conserved region, end A-like domain (aa48-241), S-S aa234, α-helix aa237-240
tp30	7%	Contains proposed adhesive domains EPLDV (aa291-295) and RGD (aa307-309)
tp31	9%	Contains RGD cell recognition sequence
tp37	9%	Contains PNP repeats
tp38	9%	Predicted α-helix (aa381-387)
tp40	9%	Predicted α-helix (aa399-405)
tp43	5%	Highly polymorphic
tp47	9%	Conserved across most strains
tp51	14%	Conserved in Africa, transmembrane (aa512-540), α-helix (aa511-512), β-sheet (aa515-519)

Details of the 14 TRAP tp peptides chosen for the cross-sectional analysis of ELISPOT responses in 217 Kenyan donors. The frequency at which they were positive in the study of 65 Kenyan adults is given, since the peptides were selected on the basis of this pilot study (see Chapter 5, Table 5.7). The amino acid sequences for each peptide can be found on Table 5.1 (Chapter 5).

6.2.4

Follow up for Malaria

Active follow up was performed weekly by a team of field workers. Each household was visited, and the temperature of each trial participant was recorded. A history was taken regarding fever and any other clinical symptoms over the preceding week. Those with a history of fever, or a temperature of >37.5°C on the day of the visit, were referred to the clinic and had duplicate finger prick blood smears performed (thick and thin films). All data was entered into a computer database using Fox-Pro software.

6.2.5

Statistical Methods

6.2.5.1

Age Group Comparisons

There were only small numbers of responders in each year group, so in certain cases the donors were divided into larger age groups: 0-3 years (n = 47), 4-6 years (n = 46), 7-9 years (n = 49), 10-14 years (n = 33), ≥15 years (n = 42). The rationale behind these subdivisions was that most episodes of clinical malaria in Northern Kilifi occur in the first 3 years of life (0-3 years), the clinical infection rate falls to almost zero by 6 years of age (4-6 years) (Gupta *et al.*, 1999). The next group was again 3 years (7-9 years), the next pre-adulthood (10-14 years), and the final group was the adults (≥15 years). All these groups contained approximately the same number of donors, and from an immunological point of view might be expected to behave differently. For some of the protection analyses the donors were divided into 3 groups only: 0-6 years, 7-15 years, ≥15 years ie peak malaria, pre-adulthood, and adults.

The number of responders in the different age groups were compared using standard chi squared analysis. Comparisons of the magnitude of the T cell responses (specific cells / 10^6 PBMC) by age were performed using student's t-tests assuming unequal variances with age as a continuous variable, and student's t-tests were used to compare between particular age groups.

6.2.5.2

Analysis for Protection

Statistical analysis for protection was performed by Amanda Ross, the statistician in Kilifi, since the data is stored on a data base in Kilifi and is confidential at present. Analysis was performed using the STATA version 6 (STATA Corporation, TX, USA) computer programme. The endpoints for the analysis were chosen before the analysis began. Analysis beyond 6 months was deemed unlikely to correlate with protection since the ELISPOT is most likely a function of recent memory, rather than long term memory (see Chapter 3). Indeed, there is evidence that malaria specific *ex-vivo* ELISPOT responses wane after 1 month (Lalvani *et al.*, 1999).

Many episodes of parasitaemia in this population were likely to be asymptomatic, and a minimum parasitaemia cut off is regarded as more likely to detect only clinically relevant cases. There is much debate surrounding this ideal cut off, and the value is likely to be specific to the malaria endemic area in question. Parasitaemias of between 400 and 20,000 parasites/μl have been proposed in previous studies (Beadle *et al.*, 1995, Färnert *et al.*, 1999), and >10,000 parasites/μl was selected for this study as likely to detect clinically relevant episodes. The analysis for protection was therefore carried out for the following 3 parameters:

Number of donors with parasitaemia over 2 months (any parasitaemia and $>10^4$/μl)

Number of donors with parasitaemia over 6 months (any parasitaemia and $>10^4$/μl)

A delay in time to first parasitaemia (any parasitaemia and $>10^4$/μl)

Fisher's exact tests were used to compare the proportions of donors with at least one episode of parasitaemia who had specific TRAP epitope T cell responses with those who did not have the response (Results 6.3.4 and 6.3.5). The mean total SFU values were compared for those who subsequently did and did not have an episode using two sample t-tests (see Results 6.3.6).

Kaplan-Meier estimates of the proportion of donors remaining episode free were plotted against time following the cross-sectional analysis of TRAP ELISPOT responses. Log-rank tests were used to compare survival experience for those donors with particular TRAP T cell epitope responses to those without (Results 6.3.4 and 6.3.5). Maentel-Haenzel tests for homogeneity were used to assess whether there was evidence of a significantly different effect of having a TRAP response in different age groups (Results 6.3.5).

The relationship between total SFU and time to first parasitaemia appeared to be non linear, thus total values were divided into 4 categories (< 0, 0-49, 50-99, ≥ 100) to look for Kaplan-Meier survival, rather than assessed as a continuous variable (Results 6.3.6). Cox's proportional hazards were used for analysis of total SFU and time to first episode of parasitaemia. The estimated effect of each total SFU category was presented as an estimated hazard ratio for an individual in each category compared to the baseline group (those with total SFU <0). It was assumed for this analysis that the effect of total SFU remained constant with time. A hazard ratio <1 meant that the hazard was less than the baseline category, and >1 meant greater than the baseline group. Differences in time to first episode between the categories were investigated using a likelihood ratio test, both univariately and after adjusting for age.

6.3
RESULTS
6.3.1
Constant Response Rate to TRAP T Cell Epitopes Maintained from 1 Year to Adulthood Despite Continuous Malaria Exposure

The 217 donors were tested in an overnight IFN-γ ELISPOT assay for reactivity to the 10 peptide conditions described above (Table 6.2), except for 68 donors in whom tp30-31 was not tested. A positive IFN-γ ELISPOT T cell response was assigned using the statistical table (described in Methods) which gives a ≥95% confidence that the response is a true positive ($p \leq 0.05$) compared to the background response. A total of 41.9% (91/217) of the donors gave a positive response to one or more of the 10 conditions, and

surprisingly only 44.2% (96/217) of the donors gave a positive PPD response (Table 6.3). The results for each of the 217 donors are tabulated in an appendix at the end of this chapter.

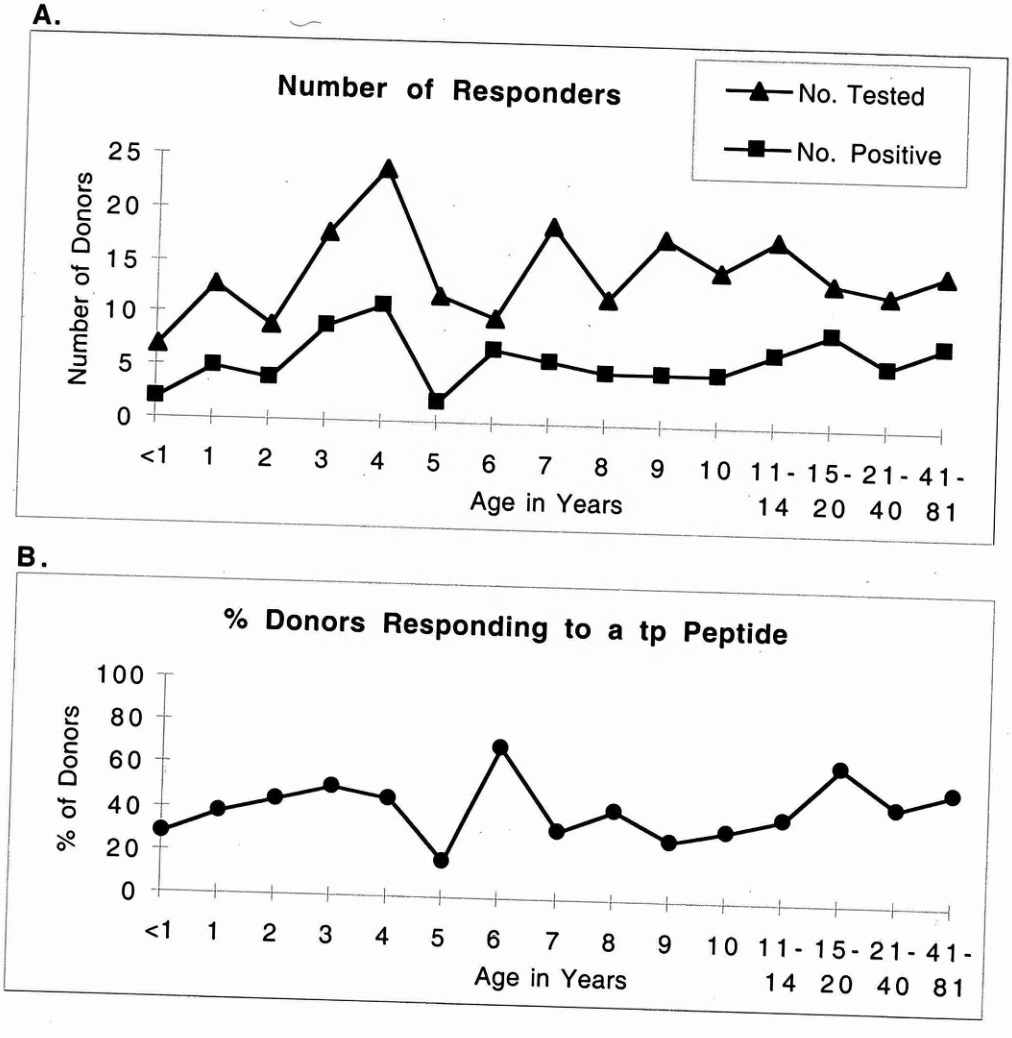
A year by year comparison of the number of donors responding to one or more peptides showed that the response rate was more or less constant throughout all age groups (Figures 6.2A and B). There was a notable dip in the 5 year olds in whom only 16.7% (2/12) responded, followed by the peak response rate of 70% (7/10) in the 6 year olds. Comparing these 2 groups suggested that the difference in proportion of responders was statistically significant (χ^2 = 6.42, p = 0.03), however this is a subgroup analysis comparing the 2 extremes over each year group, and hence is more likely to be a chance finding. Twenty nine (2/7) of the less than one year olds gave a positive peptide T cell response, which was lower than the average response rate of 42.4% (89/210) seen for the remaining donors, although this difference was not significant (χ^2 = 0.53, p = 0.7). This was not surprising given that only 7 donors were in the <1 year age group. It was interesting that by one year of age the frequency of TRAP specific T cell responses was 38.5% (5/13), a level which was then maintained more or less constant throughout all age groups (Figure 6.2B). Thus, despite continuous and repeated exposure to malaria throughout life, the frequency of donors responding did not appear to increase with exposure throughout life. This is consistent with the theory (suggested in Chapter 3) that *ex-vivo* ELISPOT responses detect effector memory T cells (T_{EM}), which are indicative of recent exposure. Thus, all donors from the same region would have the same parasite exposure, and might be expected to have comparable response rates regardless of age.

Table 6.3

AGE	n	tp2	tp4-6	tp14	tp23	tp30/1	tp37/8	tp40	tp43	tp47	tp51	PPD	TOTAL FREQ
0-3y	47	2	6	1	4	4	2	1	2	2	4	15	20
		4.3%	13.0%	2.2%	8.5%	12.9%	4.4%	2.1%	4.4%	4.4%	12.1%	32.6%	42.6%
4-6y	46	3	7	5	5	2	6	2	2	3	4	25	20
		6.5%	15.2%	10.9%	10.9%	7.1%	13.0%	4.4%	4.4%	6.5%	8.7%	54.3%	43.5%
7-9y	49	3	5	1	3	1	1	1	3	1	2	23	16
		6.1%	10.2%	1.9%	6.1%	3.3%	1.9%	1.9%	6.1%	1.9%	3.8%	46.9%	32.7%
10-14y	33	3	3	1	2	0	1	3	3	2	1	17	12
		9.1%	9.1%	3.0%	6.1%	0%	3.0%	9.1%	9.1%	6.1%	3.0%	51.5%	36.4%
≥15y	42	1	13	1	2	4	4	0	2	0	4	16	23
		2.4%	31.0%	2.4%	4.8%	11.4%	9.5%	0%	4.8%	0.0%	9.5%	38.1%	54.8%
All	217	12	34	9	16	11	14	7	12	8	15	96	91
		5.5%	15.7%	4.2%	7.4%	7.4%	6.5%	3.2%	5.5%	3.7%	6.9%	44.2%	41.9%
Adult Pilot	43	4	14	4	3	6	5	4	2	4	6	40	22
		9.3%	32.6%	9.3%	7.0%	14.0%	11.6%	9.3%	4.7%	9.3%	14.0%	93%	51.2%

Summary of all positive IFN-γ ELISPOT responses to the 10 TRAP conditions for the 217 Ngerenya donors. The details of responses for the individual donors are appended at the end of this chapter. The number of responders is the upper figure, with the percentage responders for each peptide underneath. The results for the 43 adult Kenyan donors tested to individual tp peptides spanning the whole of TRAP (Adult Pilot) is given underneath for comparison, since the 10 TRAP conditions were chosen on the basis of the responses observed in these donors.

Figure 6.2



A year by year comparison of the number of donors responding to one or more peptides showed that the response rate was more or less constant throughout all age groups (Figure 6.2A and B), and the dip in 5 year olds and peak in 6 year olds probably arose by chance.

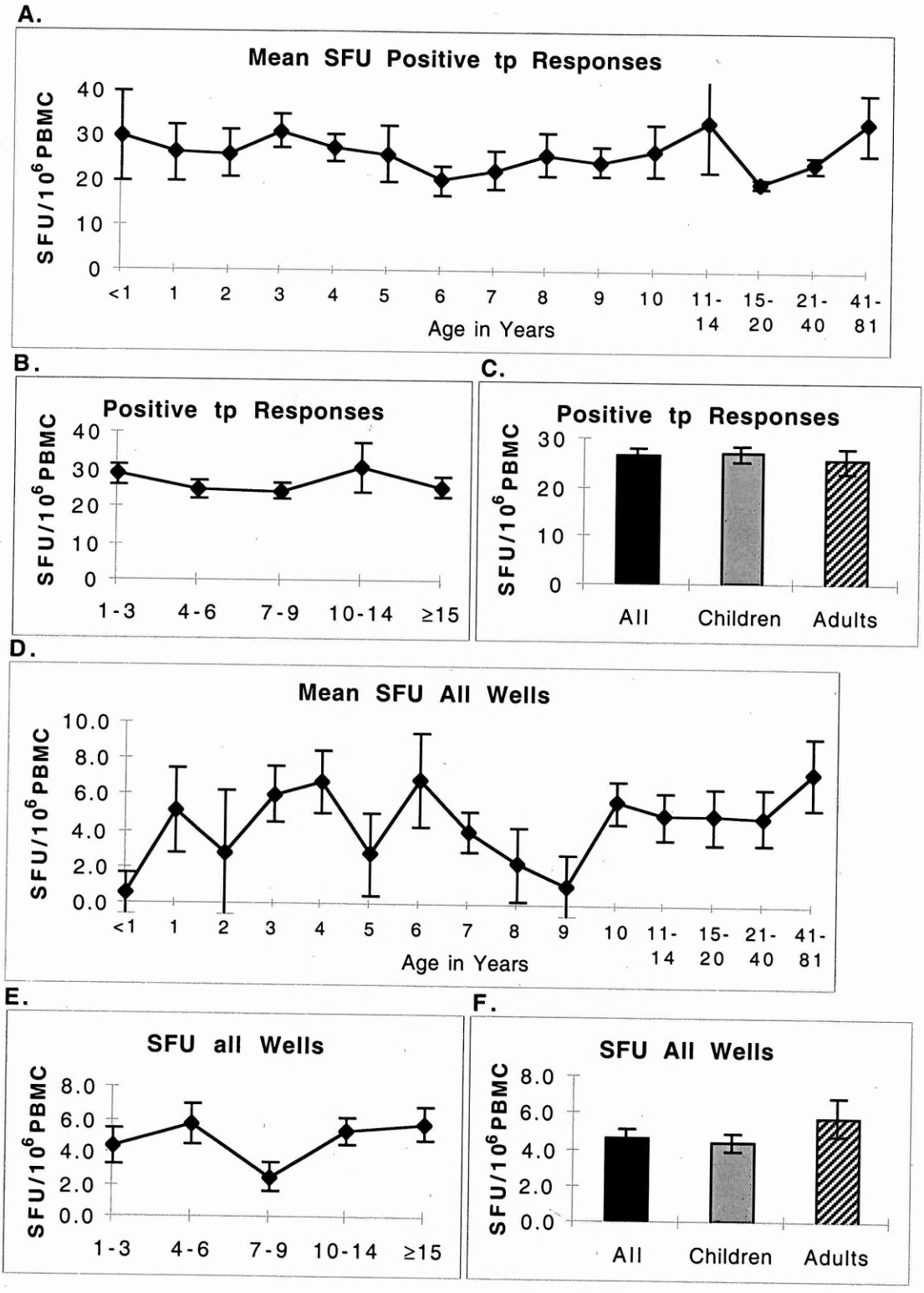
6.3.2

T Cell Precursor Frequency Levels to TRAP Epitopes in Children and Adults

The ELISPOT assay is quantitative, and gives a value for the number of cells present in the well capable of producing cytokine (as spot forming units or SFU / well) in response to the peptide(s) added to the well. A precursor frequency of specific cells per 10^6 PBMC can thus be calculated for each donor to each condition tested. The precursor frequency levels for all positive responses in this cross-sectional study averaged 26.6 ±1.4 SE / 10^6 PBMC (range 13 to 143 SFU / 10^6 PBMC). This value was comparable to those found in the Gambian (28.2 ±1.2 SE / 10^6 PBMC) and Kenyan (33.6 ±2.1 SE / 10^6 PBMC) studies detailed in chapter 5. There was no significant difference in precursor frequency levels for any of the age groups ($p = 0.53$), and even the <1 year olds averaged 30 ±10 SE SFU / 10^6 PBMC for all positive TRAP T cell responses observed (Figures 6.3 A-C). There was no significant difference in PPD precursor frequency level by age as a continuous variable ($p = 0.3$), although the <1 year olds all failed to respond to PPD (Figures 6.4 A-C), despite the fact that approximately half should have received BCG vaccination at birth (T. Mwangi, personal communication).

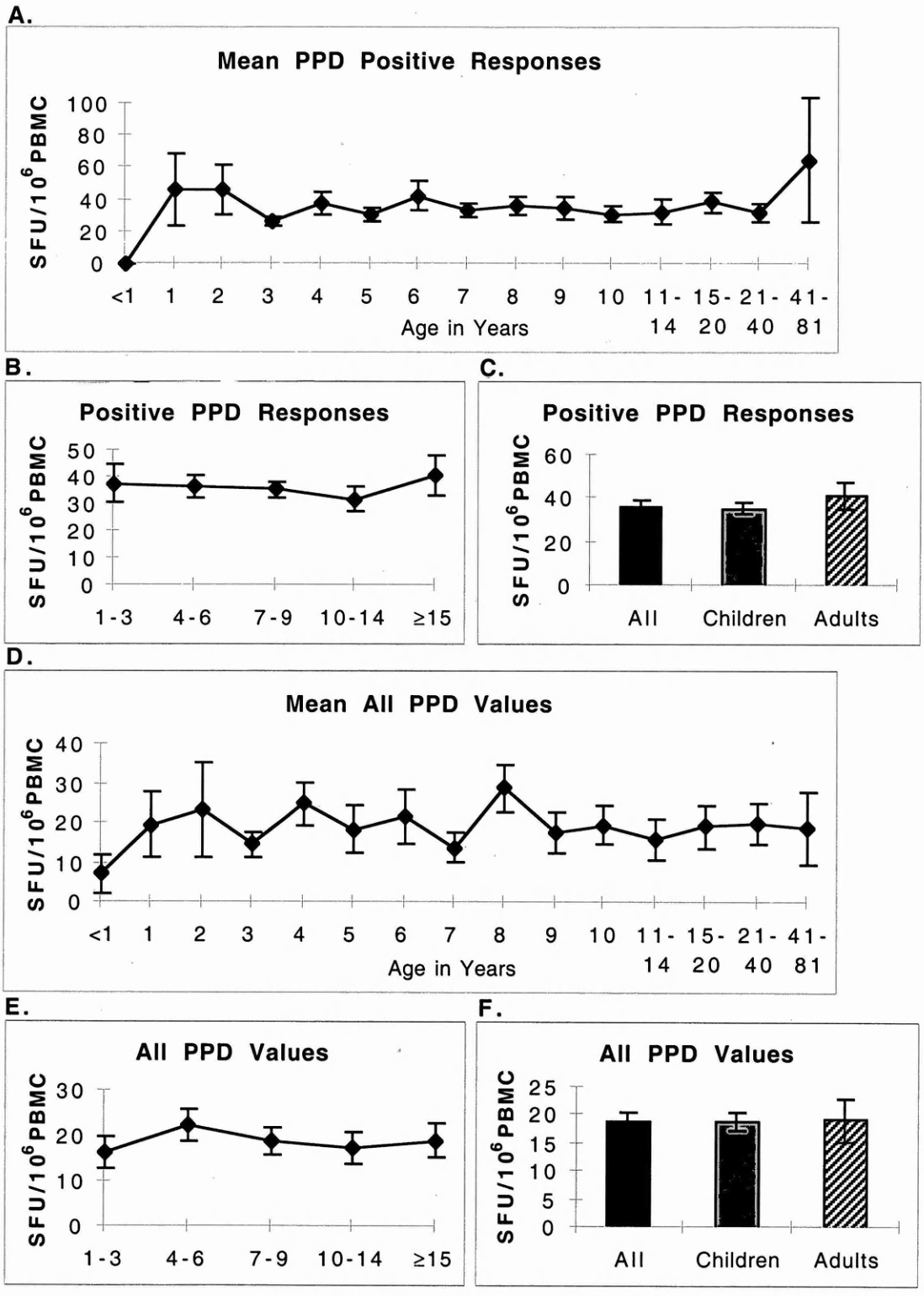
Selecting only positive responses introduces a bias in the comparison between groups since only those responses above a certain cut off are recorded positive (those with a >95% probability of being a true positive, see Methods). If all the SFU's / 10^6 PBMC are averaged for all wells, differences in precursor frequency were apparent between the age groups (Figures 6.3 D-E). The average across all age groups was 4.7 ±0.5 SE / 10^6 PBMC, and there was no significant difference by age taken as a continuous variable ($p = 0.3$). However, the <1 year olds had a lower value than the other age groups (Figure 6.3D), and a comparison with all other donors showed that responses were significantly lower ($p = 0.01$). A lower level in the <1 year olds would make sense immunologically since immunity is developing for the first time in this age group.

Figure 6.3



Precursor frequency for peptide speficic responses for positive TRAP IFN-γ responses (A-C), and responses across all wells whether positive or negative (D-E).

Figure 6.4



Precursor frequency for positive PPD IFN-γ responses only (A-C), and responses across all wells whether positive or negative (D-E).

The average SFU to TRAP peptides declined from 6 years of age, to reach a nadir in the 9 year olds (Figure 6.3D). Comparing the 9 year olds to all other donors suggested a significantly lower response rate in these donors ($p = 0.04$), but the significance was unlikely to be real since this was a non hypothesis driven subgroup analysis, and was probably a chance effect due to the small numbers in each group. Indeed, a broader subgroup analysis across the 5 age categories in Figure 6.3E showed no significant difference between age groups ($p = 0.11$), even though the 7-9 year olds still had lower levels. By 10 years of age the values had returned to the average level, and were then maintained constant throughout the adult groups (Figure 6.3D). Thus the lower precursor frequency levels observed in children compared to adults (Figure 6.3F) was accounted for by certain age subgroups, and probably the only significant difference was the lower levels in <1 year olds. The average PPD values for all wells were not significantly different when compared across age groups ($p = 0.77$), although the < 1 year olds again gave a lower value than observed for all other age groups (Figure 6.4D), although this was not significant.

6.3.3

Differences Between Adults and Children in Repertoire of T Cell Responses to TRAP

The nature of development of pre-erythrocytic immunity to malaria in naturally exposed individuals is far from understood. There is no data on pre-erythrocytic T cell responses in children assessed by ELISPOT, or indeed T cell reactivity to TRAP in childhood. I was therefore interested to see at what age the first IFN-γ ELISPOT T cell responses to TRAP would first be detected. I also wanted to assess whether naturally exposed donors would give a different repertoire of T cell responses with increasing age, or if the repertoire would remain constant in all age groups.

The earliest age at which a T cell response was seen in this study was 2 months old (donor N2 to tp4-6) (Chapter 6 Appendix), indicating that by 2 months a child's immune system is capable of developing a potentially protective T cell response to the pre-erythrocytic antigen *Pf*TRAP. I assessed for differences in repertoire of responses for the different age

groups. In a comparison of number of responders to each of the 10 TRAP conditions, tp4-6 was the most frequently positive in most age groups (Table 6.3). This was hardly surprising given that this condition consisted of a pool of 3 separate TRAP 20mers, whereas most of the other conditions contained one 20mer only. Thus, in the comparison of responses, for those conditions that consisted of >1 epitope in a well (tp4-6, tp30-1, tp37-8) the number of responders was divided by the number of epitopes added to the well to make the comparison with the other wells valid. Thus, tp4-6 responders were divided by 3, since there were 3 pooled tp epitopes added to the well.

A year by year comparison to age 10 suggested that the immunodominant T cell responses in childhood were similar between groups (Table 6.4A). Thus, tp23 and tp51 were most commonly positive. The predominant adult response was to tp4-6, although tp51 was 2nd and tp23 3rd (in addition to a number of other epitopes) (Table 6.4A). Separating the donors into larger age groups gave a similar picture to that described above, with tp23 and tp51 playing a prominent role as immunodominant responses, and tp4-6 emerging as dominant in adulthood (≥15 year olds) (Table 6.4B). The peptide pool tp30-1 was generally the least commonly recognised in most groups (Table 6.4B).

In the overall analysis for all age groups tp23 was the most frequently recognised and tp51 was second. This focus of responses on tp23 and tp51 was particularly interesting since both are conserved in Africa, and the former is highly conserved worldwide. They might thus be particularly useful vaccine components, since they would elicit cross-strain immunity. Some functional predictions have been made for these epitopes (Table 6.1), although the precise structure of TRAP remains unknown. The adult bias towards tp4-6 is also of particular interest since 2 of the constituent peptides, tp5 and tp6, contain the conserved CTL epitope tr29. In depletion studies it was found that tp4-6 can elicit both CD4 and CD8 IFN-γ ELISPOT responses, even in the same donor (Chapter 5, Figure 5.5D). It has long been thought that $CD8^+$ cytotoxic T cells play a vital role in protective immunity at the pre-erythrocytic stage of infection (reviewed in Chapter 1).

Table 6.4

A. Commonest Epitopes by Age

AGE	EPITOPE	NO. TESTED	FREQUENCY
<1y	tp51	7	1
1y	tp51	13	2
2y	tp23, 30-1, 43, 51	9	1
3y	tp23	18	3
4y	tp23	24	3
5y	tp14, 40, 47	12	1
6y	tp14, 23, 47, 51	10	2
7y	tp43	19	2
8y	tp23	12	2
9y	tp2, 43, 51	18	1
10y	tp43	15	2
11-14y	tp2, 23, 40	18	2
15-20y	tp51	14	2
21-40y	tp43	13	2
41-81y	tp4-6	15	1.7

B. Epitope Recognition Pattern by Age Group

AGE	1ST	2ND	LEAST
0-3y	tp23, 51	tp2, 4-6, 43, 47	tp14, 40
4-6y	tp14, 23	tp51	tp30-1
7-9y	tp2, 23, 43	tp51	tp30-1, 37-8
10-14y	tp2, 40, 43	tp23, 47	tp30-1
>15y	tp4-6	tp51	tp40, 47
All	tp23	tp51	tp30-1
Pilot	tp51	tp4-6	tp43

Repertoire of T cell responses to TRAP peptides varies between adults and children. NB Where there is more then one peptide per condition (tp4-6, tp30-31, tp37-38) the number of responders is divided by the number of peptides.

This focusing of the adult TRAP responses on a CTL containing T cell epitope region might be of significance since it is the adults that are semi-immune to malaria. It is possible that a bias towards CD8 T cell epitope regions might be a more generalised phenomenon, and this would need to be confirmed in a much larger study.

6.3.4

No Individual T Cell Epitope Response to TRAP Correlated With Protection

The analysis for protection was carried out to 27 weeks (just over 6 months) after the cross sectional bleed. Donors seen less than 20 times out of a possible 27 home visits (n = 13) were considered inadequately followed up, and were excluded from the study (Table 6.5A). Thus, 204 (94%) of the 217 donors recruited to the study were analysed for correlates of protection (Table 6.5B). The first question was whether IFN-γ ELISPOT responses to any of the 10 individual TRAP conditions tested (Table 6.5C) correlated with protection against the subsequent development of parasitaemia. If an individual epitope was found to be protective, this would be an ideal vaccine candidate. Analysis was performed for whether having a positive T cell epitope response by IFN-γ ELISPOT to each of the 10 conditions significantly decreased the likelihood of having an episode of parasitaemia over 2 months (10 weeks) and 6 months (27 weeks) of follow up (Table 6.6A), but no evidence for a protective effect was found (2 months p = 0.06-0.99, 6 months p = 0.14-0.99) (see Statistical Methods, 6.2.5).

Many episodes of parasitaemia in this population were likely to have been asymptomatic, and thus analysis for donors who had parasitaemias $>10^4/\mu l$ over 2 and 6 months was also performed (Statistical Methods 6.2.5). Again, there was no evidence of a significantly reduced number of episodes over 2 or 6 months in those individuals that had any of the individual tp responses (2 months p = 0.16-0.99, 6 months p = 0.14-0.99) (Table 6.6B). Kaplan-Meier analysis to 8 months was used to assess the effect of having a TRAP response on time to first parasitaemia (any parasitaemia and $>10^4/\mu l$) following the cross-sectional ELISPOT measurements. During that time 51% (104/204) of donors had ≥1 episode of parasitaemia, and 28% (58/204) had ≥1 episode of parasitaemia $>10^4/\mu l$.

Table 6.5

A. Frequency of Field Visits

No. Visits	Frequency	Percent
4-10	7	3.2
11-19	6	2.8
20	2	0.9
21	3	1.4
22	8	3.7
23	9	4.2
24	8	3.7
25	28	12.9
26	53	24.4
27	93	42.8
Total	217	100

B. Ages of Donors Analysed for Protection

Age Category	Starting No.	Excluded	Remaining	Percent
0-3 years	47	2	45	21.2
4-6 years	46	5	41	20.7
7-9 years	49	2	47	25.1
10-14 years	33	1	32	13.8
>15 years	42	3	39	19.2
Total	217	13	204	100

C. Frequency of tp Responses

	217 Donors		204 Donors	
	Freq	Percent	Freq	Percent
tp2	12	5.5	11	5.4
tp4-6	34	15.7	33	16.2
tp14	9	4.2	8	3.9
tp23	16	7.4	16	7.8
tp30-31	11	7.4	10	7.3
tp37-38	14	6.5	12	5.9
tp40	7	3.2	6	2.9
tp43	12	5.5	12	5.9
tp47	8	3.7	8	3.9
tp51	15	6.9	13	6.4

The 204 Ngerenya donors that were seen more than 20 times out of a possible 27 home visits (A) were considered adequately followed up, and were analysed for correlates of protection.

Table 6.6

A. Any Parasitaemia and Individual tp Responses

TRAP CONDITION	2 MONTHS p-value	6 MONTHS p-value
tp2	0.99	0.55
tp4-6	0.49	0.99
tp14	0.06	0.14
tp23	0.75	0.43
tp30-31	0.18	0.51
tp37-38	0.72	0.77
tp40	0.14	0.99
tp43	0.14	0.38
tp47	0.68	0.14
tp51	0.48	0.99

B. Parasitaemia $>10^4/\mu l$ and Individual tp Responses

TRAP CONDITION	2 MONTHS p-value	6 MONTHS p-value
tp2	0.6	0.55
tp4-6	0.32	0.99
tp14	0.16	0.14
tp23	0.99	0.43
tp30-31	0.5	0.51
tp37-38	0.31	0.77
tp40	0.45	0.99
tp43	0.31	0.38
tp47	0.16	0.14
tp51	0.35	0.99

Ngerenya donors were analysed over 2 and 6 months for whether any of the 10 individual TRAP tp conditions detected by IFN-γ ELISPOT correlated with protection. No significant correlates were found by Fisher's Exact Test.

Statistical analysis gave significant p-values for the tp14 and tp47 responses ($p = 0.05$ and 0.01 respectively), which suggested that having either of these responses significantly decreased the time to first parasitaemia (Table 6.7). Thus, rather than seeing a protective effect, the only statistically significant values suggested a more rapid progression to first parasitaemia for those who had that response. These results should however be interpreted with caution, since all tests were univariate, and no adjustments were made for the effect of other tp responses. In addition, this analysis involved multiple significance testing, and thus the chance of a false positive result is much greater than 5%. The effect for tp14 and tp47 was lost in the analysis for parasitaemia $>10^4/\mu l$ only (Table 6.7).

There was therefore no evidence that individual TRAP specific T cell responses provided a protective effect against the likelihood of having an episode over 2 months or 6 months, or in delaying the onset of the first episode of parasitaemia. One factor complicating this analysis was that there was no run in period to this study where parasitaemias were monitored, nor were donors treated and cleared of parasites before the study period. In retrospect this may well have been the thing to do. Therefore I do not know when each donor had their last episode of parasitaemia or clinical malaria prior to commencing the study. I do know that 48% (104/217) of donors were parasitaemic at the time of testing, comprised of 39.3% (35/89) tp responders and 63.9% (69/108) non responders. Thus, those with a positive ELISPOT response to TRAP in this study were significantly less likely to be parasitaemic at the time of testing than those with no response ($\chi^2 = 11.81$, $p = 0.006$), and conversely those with parasitaemia were less likely to have positive ELISPOT responses.

Table 6.7

Time to First Parasitaemia and Individual tp Responses

TRAP CONDITION		NO.	ANY PARASITAEMIA NO. WITH EPISODE	p-value	PARASITAEMIA >$10^4/\mu$l NO. WITH EPISODE	p-value
tp2	Neg	193	98	0.72	57	0.17
	Pos	11	6		1	
tp4-6	Neg	171	86	0.81	49	0.81
	Pos	33	18		9	
tp14	Neg	196	98	**0.05***	56	0.99
	Pos	8	6		2	
tp23	Neg	188	94	0.29	51	0.16
	Pos	16	10		7	
tp30-1	Neg	128	58	0.62	28	0.53
	Pos	10	5		3	
tp37-8	Neg	192	98	0.87	54	0.57
	Pos	12	6		4	
tp40	Neg	198	101	0.67	57	0.60
	Pos	6	3		1	
tp43	Neg	192	96	0.19	53	0.25
	Pos	12	8		5	
tp47	Neg	196	97	**0.01***	55	0.39
	Pos	8	7		3	
tp51	Neg	191	98	0.99	53	0.35
	Pos	13	6		5	

Analysis for time to first parasitaemia for donors responding to the 10 individual TRAP tp conditions detected by IFN-γ ELISPOT failed to show any significant protective effect for having a tp response. The significant values for tp14 and tp47 indicated that they cause a more rapid progression to parasitaemia. This effect was lost for the analysis for parasitaemia >$10^4/\mu$l. Log-rank survival test values given.

6.3.5 Those Donors With a TRAP Response, Compared to Those Without, Were No More Likely to be Protected

Since no individual tp epitope response correlated with protection, the next analysis addressed whether simply having an IFN-γ ELISPOT T cell response to any of the 10 TRAP conditions correlated with protection when compared to those donors who had no response. This analysis was performed for those donors who were tested by all 10 TRAP conditions only (n = 138), since 66 donors were not tested for the tp30-31 TRAP peptide pool. No significant protective effect was provided by having a TRAP specific T cell response, compared to not having one, in decreasing the chance of having an episode of parasitaemia (any or $>10^4/\mu l$) over 2 or 6 months (p-values 0.06-0.99) (Tables 6.8 A-D). Maentel-Haenzel tests for homogeneity suggested that there was no evidence of a different effect in the different age categories (p-values 0.13-0.99). Kaplan-Meier analysis suggested that having any TRAP tp response did not slow time to first parasitaemia (any or $>10^4/\mu l$) (Figures 6.5A and B). The log-rank test comparing time to first episode for those with a tp response compared to those with no response, further confirmed that there was no evidence that the presence of a response to ≥1 of the 10 TRAP conditions correlated with protection (any parasitaemia: p = 0.77 unadjusted / p = 0.28 stratified by age; parasitaemia $>10^4/\mu l$: p = 0.89 unstratified / p = 0.70 stratified).

6.3.6 Summed Response to the 10 TRAP Conditions (Total SFU) for Each Donor Failed to Correlate with Protection

Since no individual TRAP responses correlated with protection, I wondered whether the summed TRAP response to all 10 ELISPOT wells for each donor (total SFU value) would correlate with protection, since this gives a value for the total response to epitopes spanning approximately half of the TRAP antigen. Thus, for each donor, the number of spots for each well were added together, after subtracting the background negative well value for each peptide response. This gave a total precursor frequency for each donor for all 10 TRAP conditions tested. The high background responses for certain donors resulted in an overall negative total SFU value where the background response was greater than the TRAP specific responses.

Table 6.8

A. tp Response vs No tp Response: 2 Months Any Parasitaemia

	WITH tp RESPONSE			WITHOUT tp RESPONSE			
	n	FREQ PARA 2m	%	n	FREQ PARA 2m	%	p-value
0-6 y	22	9	41%	30	7	23%	
7-14 y	18	4	22%	35	1	3%	
>15 y	17	0	0%	16	0	0%	
All	57	13	23%	81	8	10%	**0.05***

Maentel-Haenzel test for homogeneity of tp response effect: p = 0.13

B. tp Response vs No tp Response: 2 Months Parasitamia $>10^4/\mu$ l

	WITH tp RESPONSE			WITHOUT tp RESPONSE			
	n	FREQ PARA 2m	%	n	FREQ PARA 2m	%	p-value
	n	$>10^4$ 2m	%	n	$>10^4$ 2m	%	p-value
0-6 y	22	3	14%	30	3	10%	
7-14 y	18	2	11%	35	1	3%	
>15 y	17	0	0%	16	0	0%	
All	57	5	9%	81	4	5%	0.49

M-H test for homogeneity: p = 0.46

C. tp Response vs No tp Response: 6 Months Any Parasitaemia

	WITH tp RESPONSE			WITHOUT tp RESPONSE			
	n	FREQ PARA 6m	%	n	FREQ PARA 6m	%	p-value
0-6 y	22	17	77%	30	13	43%	
7-14 y	18	6	33%	35	16	46%	
>15 y	17	0	0%	16	1	6%	
All	57	23	40%	81	30	37%	0.73

M-H test for homogeneity: p = 0.99

D. tp Response vs No tp Response: 6 Months Parasitaemia $>10^4/\mu$ l

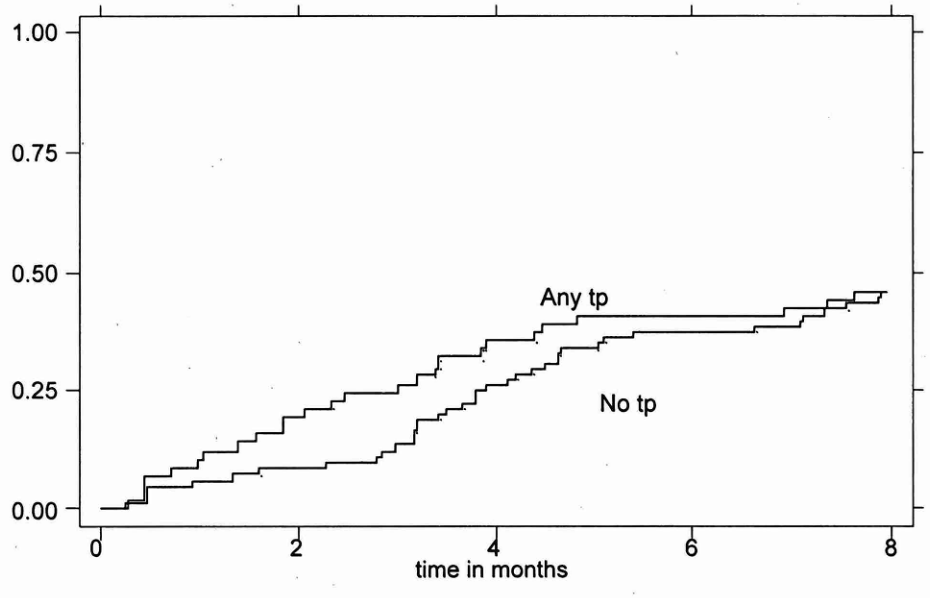
	WITH tp RESPONSE			WITHOUT tp RESPONSE			
	n	FREQ PARA 6m	%	n	FREQ PARA 6m	%	p-value
0-6 y	22	9	41%	30	9	30%	
7-14 y	18	5	28%	35	6	17%	
>15 y	17	0	0%	16	0	0%	
All	57	14	25%	81	15	19%	0.40

M-H test for homogeneity: p = 0.42

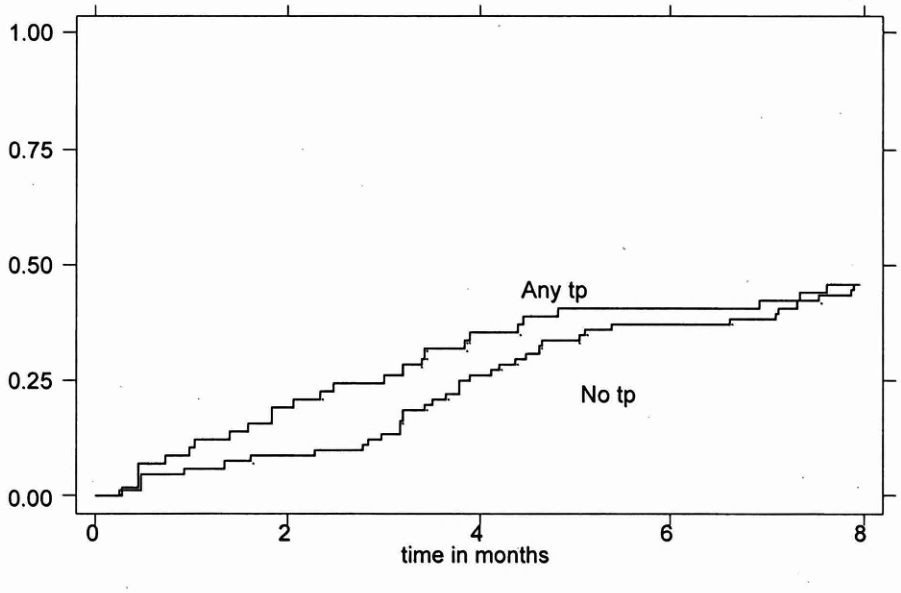
Analysis of those who had a TRAP tp response by IFN-γ ELISPOT compared to those who had none failed to show a significant protective effect.

Figure 6.5

A. TRAP tp Peptide Response Vs No tp Response Any Parasitaemia



B. TRAP tp Peptide Response Vs No tp Response Parasitaemia >$10^4/\mu l$

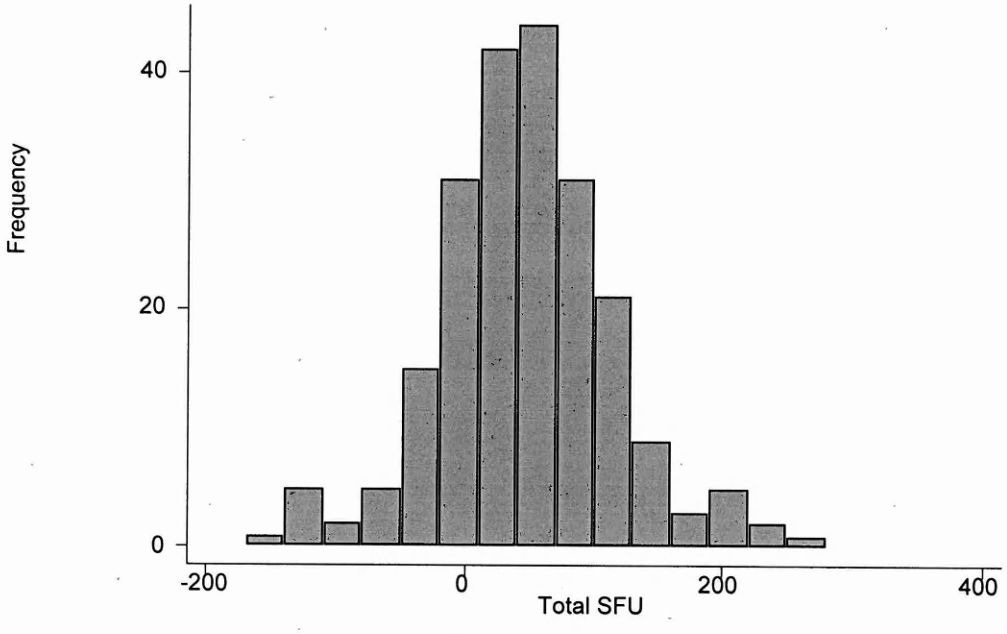


Kaplan-Meier plots for those donors who did or did not have a tp TRAP peptide response.

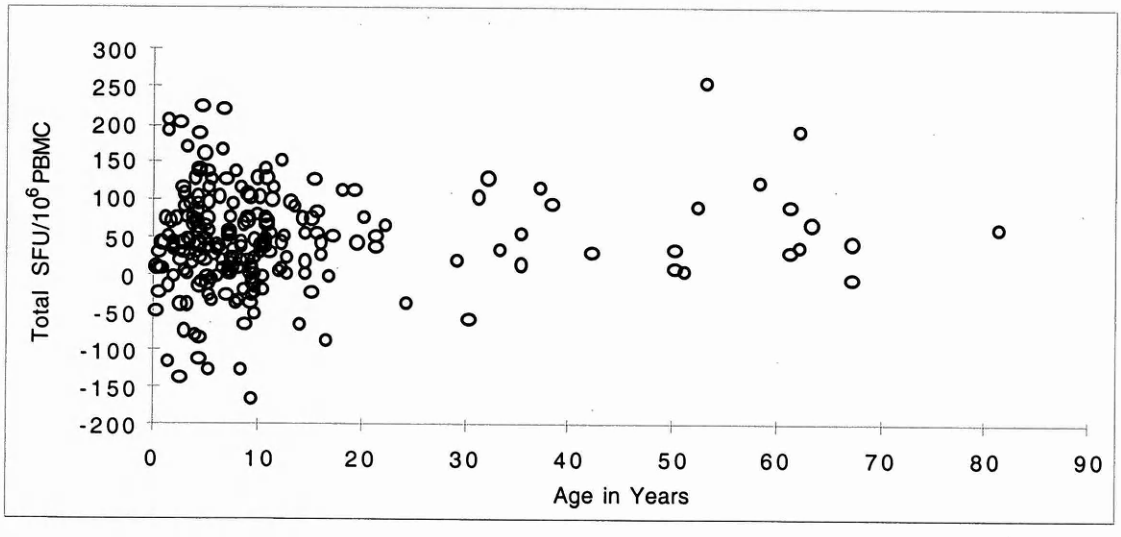
The total SFU values ranged from -165 to 255, and were normally distributed (mean 45 +/- 5 SE / 10^6 PBMC) (Figure 6.6A). A plot of total SFU by age did not show any obvious association except that very low values rarely occurred after 20 years of age (Figure 6.6B).

Figure 6.6

A. Total SFU distribution for 217 donors



B. Total SFU by Age



Analysis of whether greater total SFU values, and thus more T cells capable of responding to the 10 TRAP conditions, protected against the development of parasitaemia over 2 and 6 months was performed (Tables 6.9A and C). There was no significant difference in the total SFU values for those donors who developed parasitaemia, and those who did not (2 months $p = 0.11$; 6 months $p = 0.57$). Analysis for total SFU and the development of parasitaemia $>10^4/\mu l$ over 2 and 6 months also failed to show a significant protective effect (2 months $p = 0.78$; 6 months $p = 0.69$) (Tables 6.9B and D). Thus, there was no evidence to suggest that higher total SFU values were associated with a decrease in the number of donors with an episode of parasitaemia over 2 or 6 months, and adjusting for age had no effect on this result (not shown).

The relationship between the total SFU value and the time to first episode appeared to be non-linear, and thus the total SFU values were divided into 4 groups for analysis: <0, 0-49, 50-99, >100. Kaplan-Meier analysis for each of these 4 categories for time to 1st parasitaemia, and parasitaemia $>10^4/\mu l$ (Figures 6.7A and B). Cox's proportional hazards were used to analyse for time to first episode. The estimated effect of each total SFU category was given as a hazard ratio (see Statistical Methods 6.2.5). No significant effect was found for any of the total SFU categories and protection against the subsequent development of parasitaemia (any parasitaemia $p = 0.32$; parasitaemia $>10^4/\mu l$ $p = 0.26$) (not shown). The estimates of the hazards ratios were adjusted for age category to look for an effect of age, but none was found (any parasitaemia $p = 0.46$; parasitaemia $>10^4/\mu l$ $p = 0.68$) (not shown).

Table 6.9

A. Total SFU and Any Episode in 2 Months

	ANY EPISODE IN 2 MONTHS			NO EPISODE IN 2 MONTHS			
	n	MEAN TOT SFU	SE	n	MEAN TOT SFU	SE	p-value
0-6 y	33	52.4	15.5	53	46	9.7	
7-14 y	10	87.5	9.4	69	29.6	6.8	
>15 y	0	NA	NA	39	57.8	10.0	
All	43	60.5	12.3	161	41.8	5.0	0.11

B. Total SFU and Parsitaemia $>10^4/\mu l$ in 2 Months

	ANY EPISODE IN 2 MONTHS			NO EPISODE IN 2 MONTHS			
	n	MEAN TOT SFU	SE	n	MEAN TOT SFU	SE	p-value
0-6 y	14	41.0	27.2	72	49.9	8.6	
7-14 y	5	74.8	17.1	74	34.4	6.6	
>15 y	0	NA	NA	39	57.8	10.0	
All	19	49.9	20.6	185	45.3	4.8	0.78

C. Total SFU and Any Episode in 6 Months

	ANY EPISODE IN 6 MONTHS			NO EPISODE IN 6 MONTHS			
	n	MEAN TOT SFU	SE	n	MEAN TOT SFU	SE	p-value
0-6 y	53	55.0	11.7	33	37.9	11.0	
7-14 y	38	43.7	9.9	41	30.7	8.3	
>15 y	1	-88.0	ND	38	61.6	9.5	
All	92	48.7	8.0	112	43.3	5.6	0.57

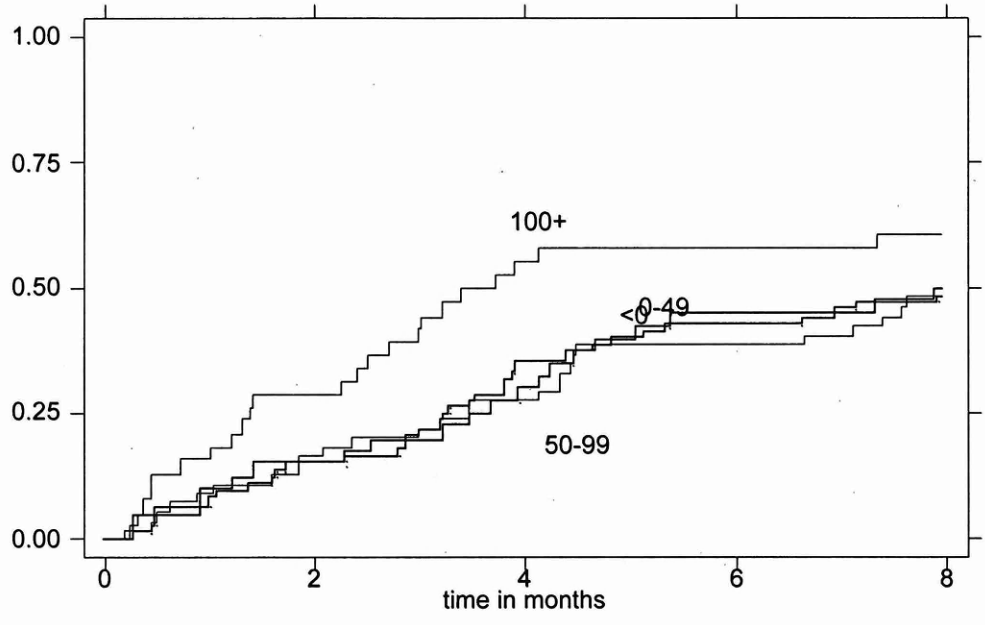
D. Total SFU and Parasitaemia $>10^4/\mu l$ in 6 Months

	ANY EPISODE IN 6 MONTHS			NO EPISODE IN 6 MONTHS			
	n	MEAN TOT SFU	SE	n	MEAN TOT SFU	SE	p-value
0-6 y	36	50.3	13.7	50	47.1	10.6	
7-14 y	15	48.9	19.1	64	34.8	6.6	
>15 y	0	NA	NA	39	57.8	10.0	
All	51	49.0	11.1	153	44.7	5.1	0.69

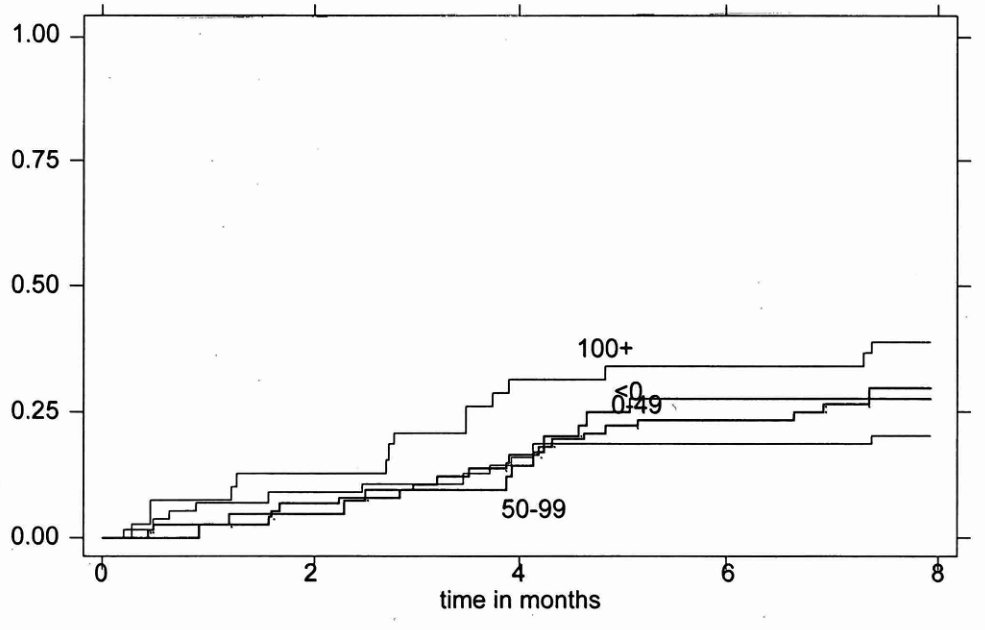
Analysis for whether the magnitude of the total SFU value to all 10 TRAP tp conditions measured by IFN-γ ELISPOT showed no protective effect over the ensuing 2 and 6 months of follow up.

Figure 6.7

A. Total SFU Any Parasitaemia



B. Total SFU Parasitaemia $>10^4/\mu l$



Kaplan-Meier plots for those donors who did or did not have a tp TRAP peptide response.

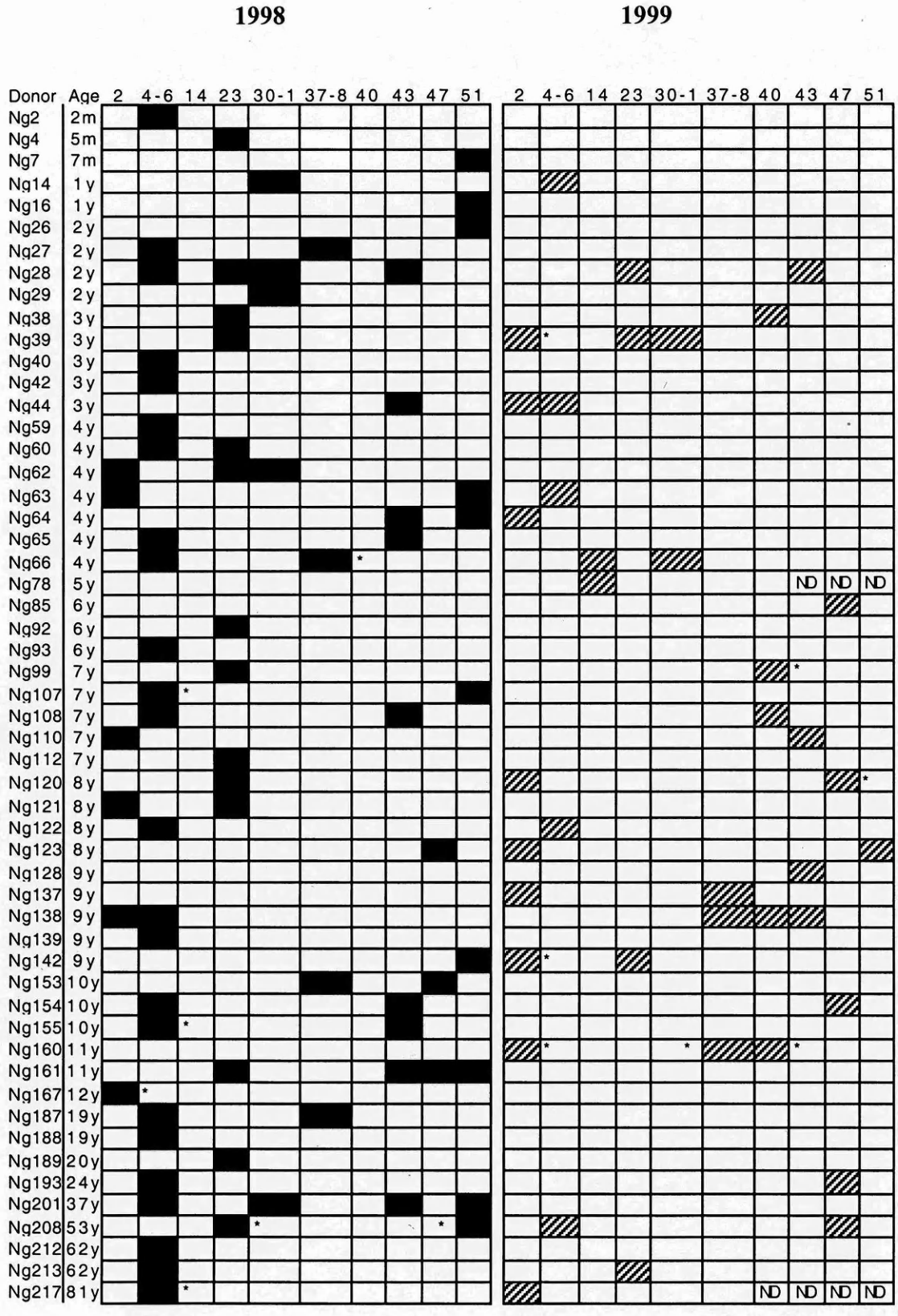
6.3.7

ELISPOT Responses Over 1 Year

I suggested in chapter 3 that *ex-vivo* ELISPOT responses reflect recent memory, or effector T cell (T_{EM}), responses. It is not known how long such memory responses persist in individuals, and no study has assessed for the change in malaria specific ELISPOT responses in naturally exposed donors over time. Thus, 69 of the 217 donors tested by IFN-γ ELISPOT assay in August / September 1988, were re-tested to exactly the same conditions in September 1999. Surprisingly, the response pattern had entirely changed over the one year period, and only 4 of the 75 positive T cell responses to TRAP detected by ELISPOT in 1998 were still present in 1999 (donors N23, N62 & N142) (Table 6.10). Even those responses that were very high in 1998 (marked with an asterisk) were no longer present on re-testing in 1999, and conversely the high values in 1999 were not present on testing in 1998. In the 1998 analysis, tp23 was the most frequently recognised epitope, being positive 20.6% (14/68) donors, and tp51 was second in 14.7% (10/68) of donors. In 1999 the response rates to tp23 and tp51 were 5.9% (4/68) and 1.5% (1/66 tested) respectively. The most frequently positive response in 1999 was to tp2 (9/68, 13.2% of donors), all but one of which occurred in children (<15 years of age). Thus, once again the most frequently recognised epitope was conserved. Since tp2, tp23 and tp51 are all conserved epitopes then the fact that the repertoire has changed over the year cannot be a result of exposure to different parasite strains, since all strains will carry the same conserved regions. The change must be a result of other factors. The number of adult donors that responded in this study was too small (4/68) to draw any conclusions regarding T cell repertoire differences in adults.

IFN-γ ELISPOT responses to *Pf*TRAP T cell epitopes were therefore not retained over a one year period. I do not know how long such T cell responses persist in malaria exposed individuals, and this will require a longitudinal follow up study with repeated testing. The short lived nature of these responses may be the reason that no correlates of protection were found in this study.

Figure 6.8



*Repeat IFN-γ ELISPOT testing after 1 year led to a complete change in the pattern of responses for each donor. * indicates very high ELISPOT responses ($p<0.01$).*

It is possible that any protective effect afforded by T cell responses detectable by *ex-vivo* ELISPOT is very short lived ie up to a month, although a delay in time to first parasitaemia might have been expected in this study in the younger age groups if that were the case.

The response rate in 1999 of 36.8% (25/69) was much lower than the response rate of 70.6% (48/68) in the same donors the previous year. The probable explanation for this is selection bias, since predominantly TRAP responders were selected from the 1998 cohort in order to address the question of whether responses were maintained. The overall response rate in the 1998 study of 41.9% (91/217) was comparable to the rate of 36.8% obtained in 1999. The positive PPD response rate of 46.4% (32/69) in 1999 was comparable to the 40.6% (28/69) who were positive in 1998, with 4 new cases arising in the 0-1 year old donors (age in 1998). The PPD batches used were different for the 2 studies, and the unexpectedly low reactivity rate of 50% was even observed for the adults tested. Thus, a PHA response was tested in 1999 as a second positive control and 100% of donors gave a positive response, confirming that the assay was working for all donors. The magnitude of positive responses (precursor frequency level) seen on re-testing in 1999 could not be compared to 1998 because of the significantly higher background levels in 1999

The 69 donors that were re-tested in 1999 were followed up weekly throughout the year preceding the analysis. The number of malaria episodes seen in the preceding 12 months for the whole group was 2.3 _0.2 SE for all parasitaemias, and 1.4 _0.2 SE for parasitaemia >104/µl. As expected the majority of cases occurred in the younger age groups (0-6 years). Since it is believed that ex-vivo ELISPOT responses detect recent memory, I looked for a statistical association between the presence of a positive ELISPOT response in 1999 and episodes of parasitaemia (any and >104/µl) in the preceding 1 to 5 months. There was no evidence to suggest that those donors who had a positive TRAP IFN-_ ELISPOT response in 1999 were more likely to have had an episode of parasitaemia (any or >104/µl) in the previous months than those who did not (_2 analysis 0.04-1.86; p-value range 0.17-0.85).

Thus, the presence of a TRAP IFN-_ ELISPOT response to ≥1 of the 10 conditions was not significantly associated with parasitaemia in the 5 months prior to ELISPOT testing.

6.4

DISCUSSION

The development of pre-erythrocytic immunity in naturally exposed donors is far from understood. No study has comprehensively compared immune responses in adults and children for TRAP, or indeed any other pre-erythrocytic antigen. Thus, IFN-γ ELISPOT responses were assessed to 14 TRAP epitopes spanning half the molecule for 217 Kenyan donors aged between 2 months to 81 years. All donors were from a single study area called Ngerenya in Northern Kilifi, and thus had similar HLA types, comparable rates of parasite exposure, and bed net usage.

Forty two percent of donors overall responded to one or more of the 10 TRAP T cell conditions tested in the study. A response rate of 28.6% was observed for the <1 year olds, and the reactivity rate of 38.5% in the one year olds was similar to the reactivity levels observed in adults. Thus the overall response rate of approximately 40% was established by one year of age, and was comparable to the reactivity in adults despite the fact that the latter had experienced continuous and repeated lifelong exposure to malaria. The precursor frequency level for positive T cell ELISPOT responses to TRAP epitopes was also constant, even in the <1 year olds. This may not be a valid comparison since only those wells reaching a certain threshold were considered positive. Indeed, when the responses for all wells were averaged, the lower level observed in the <1 year olds was significantly different to the rest of the donors. A decline in levels was also observed from 7 to 9 years, but was not statistically significant. It is easy to understand why the <1 year olds would have lower T cell precursor frequencies, since immunity is just beginning to develop in them as a result of exposure in the first year of life. It is useful to know that children as young as one year develop T cell responses to TRAP that might be susceptible to boosting by vaccination.

The T cell repertoire differences between adults and children were interesting, since the predominant childhood responses focused on two conserved CD4 T cell epitopes (tp23 and tp51) in 1998, and on another highly conserved epitope (tp2) upon re-testing in 1999. The immunodominant epitope in the adults was tp4-6, even after allowing for the fact that this consisted of 3 individual epitopes tp4, tp5, and tp6. The conserved HLA A2 restricted CTL epitope tr29 is contained with tp5 and tp6, and it is possible that the adults focused their responses on the CTL epitope within these peptides. CD4 and CD8 T cell depletion studies were not performed as part of this study, but I do know that this peptide pool can elicit both CD4 and CD8 T cell responses, even in the same donor. A future study might address whether adults and children do indeed focus on different conserved regions within TRAP, and whether adults preferentially select conserved CTL epitope regions. The focus of T cell reactivity towards conserved T cell epitope regions within TRAP found in this study was completely different to CS, where the immunodominant T cell epitopes are all concentrated in polymorphic regions within the carboxy terminus of the antigen (reviewed in Chapter 1). This might suggest that TRAP is a more suitable vaccine candidate than CS, since conserved regions will provide cross-strain protection.

Analyses for correlates of protection for T cell responses to pre-erythrocytic antigens have been disappointing. Two studies hinted at protection mediated by a single immunodominant CD4 T cell epitope region of CS (Hoffman *et al.*, 1989b, Riley *et al.*, 1990), and one study showed protection to a peptide determinant in LSA-1 (Luty *et al.*, 1999). Most studies have focused on lymphoproliferative responses to a few peptide determinants, but given the evidence that the IFN-γ ELISPOT might detect protective responses, I speculated that it might provide correlates of protection in malaria exposed Kenyans. Since reactivity to TRAP was so high in Kenyans, I investigated whether IFN-γ ELISPOT T cell responses to TRAP derived peptides correlated with protection for the 217 Kenyan donors assayed in 1998. IFN-γ ELISPOT responses probably detect recent memory / effector memory T cells (Chapter 3) and I predicted that a protective effect beyond 6 months was highly unlikely. Thus, the 204 donors who were followed up

adequately were analysed for correlates of protection over 2 months, and 6 months, in addition to time to first parasitaemia.

The analysis assessed for the presence of a significant protective effect by any of the 10 individual TRAP conditions; total response across all 10 wells (total SFU); and whether having any ELISPOT response to TRAP compared to none at all was protective. Unfortunately, none of the analyses showed a protective effect for TRAP specific IFN-γ ELISPOT T cell responses. The possible reason(s) for this lack of protection are numerous. The IFN-γ ELISPOT assay may not measure T cell responses that are protective against malaria. There is evidence from vaccine trials that malaria specific T cell responses measured by *ex-vivo* ELISPOT responses wane after one month (Lalvani *et al.*, 1999), and in this study cultured ELISPOT responses were shown to be more durable. Perhaps cultured responses are more important in protective immunity than the overnight unstimulated *ex-vivo* responses. Indeed, a study of naturally exposed Gabonese children showed that cultured (day 6) IFN-γ production to peptides derived from the liver stage antigen (LSA-1) were associated with significantly delayed first reinfections and significantly lower rates of reinfection in those children admitted with mild malaria (Luty *et al.*, 1999), and this is the only human study linking IFN-γ production with protection at the pre-erythrocytic stage in naturally exposed donors. The *ex-vivo* ELISPOT responses may be too short lived to have found a significant protective effect in the study in this chapter. However, if a protective effect did exist it might have manifested in a delay in time to first parasitaemia in the younger age groups.

The power for detecting a protective effect for TRAP responses in this study was determined retrospectively, since I did not know at the onset of the trial how many donors would respond, and what proportions would respond in the different age groups. For the individual peptide analysis estimates in the region of 25% power have been made, and thus increasing the number of donors might have improved the chances of seeing protection. None of the results stand out as being close to protective, and in certain cases the trend went in the 'wrong' direction eg the presence of a tp14 or tp47 response decreased time to

first parasitaemia. In these latter cases, increasing the number of donors and power would have been insufficient to demonstrate protection.

I may not have chosen the optimal end points for the analysis. For example, it is possible that parasitaemias $>10^4/\mu l$ were not the best cut off to analyse for, and that a value of $>10^3/\mu l$ might have been better. Alternatively, the analysis may have shown protection if analysis was for the first 6 weeks, rather than 10 weeks, of follow up. Further analyses may show a protective effect, although the p-value of such an effect would have to be highly significant for it to be convincing, since the multiple analyses performed thus far increase the likelihood of a false positive result. Further studies would then be required to confirm any significant protective effect found on re-analysis of this data. Another possibility that could explain the lack of protection is that TRAP specific T cell responses are not protective in humans. This seems unlikely given that TRAP has such an important functional role at the pre-erythrocytic stage of infection, including sporozoite motility and hepatocyte invasion (Müller *et al.*, 1993, Sultan *et al.*, 1997) (reviewed in Chapter 1). Moreover, high levels of protection against subsequent malaria challenge can be achieved in mice immunised with constructs expressing the whole of TRAP (Schneider *et al.*, 1998).

The development of pre-erythrocytic immunity to malaria is far from understood, and is likely to be a complex response to multiple B cell and T cell (CD4 and CD8) epitopes to multiple antigens. The data in this chapter provides some insights into the nature of the development of pre-erythrocytic immunity, and further illustrates the complexity of T cell immunity at this stage of infection. A child of 2 months of age was capable of mounting an IFN-γ T cell response to TRAP, and by one year of age the levels of reactivity were comparable to those seen in adults, both in terms of absolute numbers of responders and precursor frequency levels achieved. Differences in T cell repertoire between adults and children were observed, and further investigation of this may provide an insight into why adults, but not children, enjoy a state of partial immunity. The fact that a protective effect was not observed for IFN-γ ELISPOT T cell responses to TRAP in this study does not

mean T cell responses to this antigen are not protective. This assay still warrants further analysis for protective correlates, as do T cell responses to TRAP.

6.5

APPENDIX

Summary of All ELISPOT Responses in Cross-Sectional Bleed

IFN-γ ELISPOT Responses TRAP Peptides by Age for Donors Ng1 - Ng217

DONOR	AGE	tp2	tp4-6	tp14	tp23	tp30-1	tp37-8	tp40	tp43	tp47	tp51	PPD
Ng1	1 m											
Ng2	2 m											
Ng3	4 m											
Ng4	5 m											
Ng5	5 m											
Ng6	7 m											
Ng7	9 m					NT						
Ng8	1					NT						
Ng9	1											
Ng10	1											
Ng11	1											
Ng12	1											
Ng13	1											
Ng14	1											
Ng15	1											
Ng16	1					NT						
Ng17	1											
Ng18	1					NT						
Ng19	1											
Ng20	1					NT						
Ng21	2					NT						
Ng22	2											
Ng23	2											
Ng24	2											
Ng25	2											
Ng26	2											
Ng27	2					NT						
Ng28	2											
Ng29	2											
Ng30	3											
Ng31	3					NT						
Ng32	3					NT						
Ng33	3					NT						
Ng34	3					NT						
Ng35	3											
Ng36	3					NT						
Ng37	3					NT						
Ng38	3											
Ng39	3					NT						
Ng40	3											
Ng41	3											
Ng42	3											
Ng43	3											
Ng44	3					NT						
Ng45	3											
Ng46	3											
Ng47	3					NT						

p < 0.05
p < 0.01

Positive responses are indicated by filled in squares. A positive score was allocated according to the statistical table in Methods (Section 2.16.1)

Responses to tp TRAP Peptides by Age continued:

DONOR	AGE	tp2	tp4-6	tp14	tp23	tp30-1	tp37-8	tp40	tp43	tp47	tp51	PPD
Ng48	4					NT						
Ng49	4					NT						
Ng50	4					NT						
Ng51	4											
Ng52	4											
Ng53	4					NT						
Ng54	4					NT						
Ng55	4					NT						
Ng56	4					NT						
Ng57	4											
Ng58	4											
Ng59	4					NT						
Ng60	4											
Ng61	4					NT						
Ng62	4											
Ng63	4											
Ng64	4					NT						
Ng65	4					NT						
Ng66	4											
Ng67	4											
Ng68	4											
Ng69	4											
Ng70	4											
Ng71	4											
Ng72	5					NT						
Ng73	5					NT						
Ng74	5					NT						
Ng75	5					NT						
Ng76	5											
Ng77	5											
Ng78	5											
Ng79	5											
Ng80	5					NT						
Ng81	5					NT						
Ng82	5					NT						
Ng83	5											
Ng84	6											
Ng85	6											
Ng86	6											
Ng87	6											
Ng88	6											
Ng89	6											
Ng90	6											
Ng91	6											
Ng92	6											
Ng93	6											

p < 0.05
p < 0.01

Positive responses are indicated by filled in squares. A positive score was allocated according to the statistical table in Methods (Section 2.16.1)

Responses to tp TRAP Peptides by Age continued:

DONOR	AGE	tp2	tp4-6	tp14	tp23	tp30-1	tp37-8	tp40	tp43	tp47	tp51	PPD
Ng94	7											
Ng95	7											
Ng96	7											
Ng97	7											
Ng98	7					NT						
Ng99	7											
Ng100	7					NT						
Ng101	7					NT						
Ng102	7											
Ng103	7											
Ng104	7											
Ng105	7											
Ng106	7											
Ng107	7											
Ng108	7					NT						
Ng109	7											
Ng110	7					NT						
Ng111	7					NT						
Ng112	7											
Ng113	8											
Ng114	8					NT						
Ng115	8					NT						
Ng116	8											
Ng117	8											
Ng118	8											
Ng119	8					NT						
Ng120	8					NT						
Ng121	8					NT						
Ng122	8											
Ng123	8					NT						
Ng124	8											
Ng125	9					NT						
Ng126	9					NT						
Ng127	9					NT						
Ng128	9											
Ng129	9											
Ng130	9											
Ng131	9											
Ng132	9					NT						
Ng133	9											
Ng134	9											
Ng135	9											
Ng136	9											
Ng137	9					NT						
Ng138	9					NT						
Ng139	9					NT						
Ng140	9											
Ng141	9											
Ng142	9											

p < 0.05
p < 0.01

Positive responses are indicated by filled in squares. A positive score was allocated according to the statistical table in Methods (Section 2.16.1)

Responses to tp TRAP Peptides by Age continued:

DONOR	AGE	tp2	tp4-6	tp14	tp23	tp30-1	tp37-8	tp40	tp43	tp47	tp51	PPD
Ng143	10											
Ng144	10											
Ng145	10											
Ng146	10											
Ng147	10											p < 0.05
Ng148	10											p < 0.01
Ng149	10											p < 0.05
Ng150	10											p < 0.05
Ng151	10					NT						p < 0.05
Ng152	10							p < 0.05				p < 0.05
Ng153	10						p < 0.05			p < 0.05		
Ng154	10		p < 0.05						p < 0.05			p < 0.01
Ng155	10		p < 0.01						p < 0.05			
Ng156	10	p < 0.05										p < 0.01
Ng157	10											p < 0.01
Ng158	11											
Ng159	11					NT						p < 0.05
Ng160	11				p < 0.05							p < 0.05
Ng161	11	p < 0.01				NT						
Ng162	12											
Ng163	12					NT						p < 0.05
Ng164	12											p < 0.05
Ng165	12											p < 0.05
Ng166	12					NT						p < 0.05
Ng167	12				p < 0.05				p < 0.05	p < 0.05	p < 0.05	p < 0.05
Ng168	12							p < 0.05				
Ng169	13			p < 0.05		NT		p < 0.05				
Ng170	13		p < 0.01									p < 0.01
Ng171	14	p < 0.05										
Ng172	14											
Ng173	14					NT						
Ng174	14											
Ng175	14					NT						

Positive responses are indicated by filled in squares. A positive score was allocated according to the statistical table in Methods (Section 2.16.1)

Responses to tp TRAP Peptides in Adults:

DONOR	AGE	tp2	tp4-6	tp14	tp23	tp30-1	tp37-8	tp40	tp43	tp47	tp51	PPD
Ng176	15											
Ng177	15											
Ng178	15											
Ng179	15											
Ng180	15											
Ng181	16					NT						
Ng182	16											
Ng183	16											
Ng184	16					NT						
Ng185	17											
Ng186	18											
Ng187	19											
Ng188	19											
Ng189	20											
Ng190	21											
Ng191	21					NT						
Ng192	22											
Ng193	24											
Ng194	29											
Ng195	30											
Ng196	31											
Ng197	32											
Ng198	33											
Ng199	35											
Ng200	35					NT						
Ng201	37											
Ng202	38											
Ng203	42											
Ng204	50											
Ng205	50											
Ng206	51											
Ng207	52											
Ng208	53					NT						
Ng209	58											
Ng210	61											
Ng211	61											
Ng212	62											
Ng213	62					NT						
Ng214	63											
Ng215	67					NT						
Ng216	67											
Ng217	81											

p < 0.05
p < 0.01

Positive responses are indicated by filled in squares. A positive score was allocated according to the statistical table in Methods (Section 2.16.1)

CHAPTER 7

CONCLUDING REMARKS

Throughout this thesis I have attempted to further elucidate the nature of naturally acquired T cell immunity at the pre-erythrocytic stage of malaria infection. The main purpose of this goal is to use the information to optimise the design of pre-erythrocytic stage malaria vaccines. Immunisation of animals and humans with irradiated sporozoites induces sterile heterologous protection against malaria challenge (Nussenzweig *et al.*, 1969, Clyde *et al.*, 1973, Clyde *et al.*, 1975, Rieckmann *et al.*, 1979), and the hope is that this protective effect can be mimicked by developing vaccines that stimulate anti-sporozoite and anti-liver stage immunity. The effect of irradiated sporozoites is likely to be mediated by responses to hundreds or thousands of T and B cell epitopes, however if a handful of protective T cell epitopes can be identified in key pre-erythrocytic antigens, then boosting T cell responses to artificially high levels might provide protection.

7.1

Insights Into Malaria T Cell Non-Responsiveness

One unusual factor of pre-erythrocytic stage T cell immune responses in naturally exposed healthy donors is the widespread nonresponsiveness to malaria antigens and epitopes (Good *et al.*, 1988d, Riley *et al.*, 1988b, Doolan *et al.*, 1993, Doolan *et al.*, 1994, Plebanski *et al.*, 1997a). This is observed in adults as well as children, despite the former having been repeatedly and continuously exposed to malaria throughout life. Such nonresponsiveness is malaria specific, since T cell responses to recall antigens such as PPD are generally preserved, apart from during episodes of acute malaria infection (Ho *et al.*, 1986). Indeed, the immunosuppression observed during malaria infection seems to be a separate entity from the generalised malaria nonresponsiveness seen in healthy donors (Ho *et al.*, 1988, Theander *et al.*, 1986, Riley *et al.*, 1988a, Chemtai and Okelo, 1989, Hviid *et al.*, 1991b, Hviid *et al.*, 1991a). A variety of immunological mechanisms have been proposed to cause this phenomenon including T cell tolerance induction in the neonate (Pombo *et al.*, 1988),

peripheral T cell anergy, 'original antigenic sin' (Good *et al.*, 1993), and the induction of immunosuppressive activated T cells (Riley *et al.*, 1989, Mshana *et al.*, 1990, Theander *et al.*, 1993).

7.1.1

Use of 3 T Cell Assays

The studies upon which these broad conclusions of T cell nonresponsiveness are based employed the lymphoproliferation assay when assessing for CD4 T cell reactivity (Good *et al.*, 1988d, Riley *et al.*, 1988b, Doolan *et al.*, 1994). Few of the studies of pre-erythrocytic antigens assessed for the release of cytokines in parallel to lymphoproliferation, but when they have the results have been contradictory. Thus, Riley and colleagues found no correlation between IFN-γ release (by ELISA) and proliferative responses to CS protein in Gambian children (Riley *et al.*, 1990). By contrast, Doolan and colleagues found a positive correlation between IFN-γ production (by ELISA) and proliferation in a study of CD4 T cell responses to CS protein in adults from Papua New Guinea (Doolan *et al.*, 1994).

The ELISPOT assay is a relatively new tool, and is a rapid and simple way to assess for IFN-γ responses to multiple epitopes in the field. It has never been used in field studies of natural T cell immunity, and I wondered whether it would detect the same responses as lymphoproliferation assays. Another T cell assay was also developed for my study whereby cells were first re-stimulated *in vivo* with pools of peptides, and then tested by an overnight ELISPOT assay for IFN-γ release (cultured ELISPOT). T cell responses to 8 peptides spanning the main T cell epitope regions of CS protein were assessed by all 3 assays (proliferation, *ex-vivo* ELISPOT, cultured ELISPOT) in adult Gambians. Surprisingly, none of the 3 assays correlated suggesting that the 3 assays detect different T cell subsets. The simultaneous use of 3 assays markedly increased the T cell reactivity level compared to that detectable by one assay alone. Therefore, one reason for the T cell nonresponsiveness observed by many groups may be that many reactive memory T cells are missed by using one T cell assay alone.

7.1.2

Different Memory T Cell Subsets

I proceeded to confirm that, as expected, the *ex-vivo* ELISPOT and proliferative assays were mediated by $CD45RO^{+}RA^{-}$ memory T cells. I also found that $CD45RB^{+}$ cells play a significant role in mediating *ex-vivo* ELISPOT responses. I further demonstrated that *ex-vivo* ELISPOT responses were mediated by a putative $CCR7^{-}$ effector memory (T_{EM}) T cell population, whereas cultured ELISPOT responses were generated by a $CCR7^{+}$ central memory (T_{CM}) T cell population, indicating that the two IFN-γ assays detect the different memory T cell subsets first proposed by Sallusto and colleagues (Sallusto *et al.*, 1999).

Depletion from PBMC of cells expressing the T cell activation marker CD38 (Malavasi *et al.*, 1994) led to a significant increase in lymphoproliferative responses to the recall antigen PPD in many donors tested. This effect was more marked the lower the starting proliferative response, and was not apparent if the initial PBMC proliferative response was high. This suggested that $CD38^{+}$ cells actively suppress lymphoproliferation in an antigen specific manner in certain donors. I went on to demonstrate that $CD4^{+}38^{+}$ T cells can actively suppress the proliferation of $CD4^{+}38^{-}$ T cells to PPD and the gluten derived coeliac allergen GIII; and $CD38^{+}$ PBMC can inhibit responses of 38^{-} PBMC to PPD, TT and GIII. The inhibitory effect required cell-cell contact since it did not occur when the inhibitory cells were added across transwells. Thus, the release of the classic inhibitory mediators IL-10 and TGFβ could not explain the inhibitory effect of these $CD38^{+}$ T cells. These findings are in agreement with those of Read and colleagues in the murine model who identified an $CD38^{+}CD45RB^{low}CD4^{+}$ T cell subset which inhibits proliferation of $CD38^{-}$ $CD4^{+}$ T cells by a mechanism requiring cell-cell contact (Read *et al.*, 1998). I demonstrated for a malaria exposed donor that proliferative responses to an immunodominant Th2R malaria CD4 T cell epitope were enhanced by $CD38^{+}$ cell depletion of PBMC. Thus, $CD38^{+}$ T cells may be inhibitory in malaria, and may play a role in malaria specific T cell nonresponsiveness. This will need to be explored in future studies.

7.2

Significance of Antigenic Polymorphism

African *P. falciparum* is estimated to have arisen from a single ancestral strain some 25-60 thousand years ago (Rich *et al.*, 1998), and throughout this time has developed numerous mechanisms for evading the host immune response. The immune selection pressure caused by the malaria parasite is enormous considering that it is responsible for maintaining a variety of harmful genetic disorders (sickle, thalassaemia, G6PD deficiency) because the heterozygous state is protective against malaria (Allison, 1954, Flint *et al.*, 1986, Ruwende *et al.*, 1995). Indeed, it also has a profound influence on the maintenance of MHC polymorphism in Africans (Hill *et al.*, 1991, Hill *et al.*, 1992b, Hill *et al.*, 1992a).

7.2.1

Lack of Cross-Recognition

CS protein was the first pre-erythrocytic antigen to be discovered, and there was initially great optimism that it might be an ideal vaccine candidate. One caveat is that the main T cell epitope regions are highly polymorphic (de la Cruz *et al.*, 1988b, Good *et al.*, 1988c, Good *et al.*, 1988d, de Groot *et al.*, 1989), and there was evidence that there is limited cross-recognition of polymorphic variants in naturally exposed populations (de la Cruz *et al.*, 1988a, de la Cruz *et al.*, 1989, Udhayakumar *et al.*, 1994, Zevering *et al.*, 1994). I have demonstrated in a study of Gambian adults that there is indeed limited cross-recognition of the naturally occurring variants of the immunodominant epitope Th2R of CS protein.

7.2.2

Altered Peptide Ligand Antagonism

The phenomenon of altered peptide ligand antagonism (APL) is a recognised immunoevasion strategy employed by the HIV (Klenerman *et al.*, 1994) and hepatitis B (Bertoletti *et al.*, 1994) viruses, and more recently recognised for a CTL epitope region within CS protein of malaria (Gilbert *et al.*, 1998). I found that APL also operates for a CD4 T cell epitope region within the Th2R immunodominant domain of CS protein. This is the first demonstration of APL for naturally occurring CD4 T cell epitope variants for

any infectious pathogen. The mechanism involved the switching of a single CD4 T cell clone from production of the protective cytokine IFN-γ induced by one variant, to the immunosuppressive cytokine IL-10 induced by the antagonist. This is therefore another means by which malaria immunosuppression might arise, and helps explain how variant parasites escape host immune responses. The effect is not only at the effector T cell stage, since the dual presentation of APL variants at the time of T cell priming can prevent the generation of memory T cells altogether, and thus generate functionally naïve hosts (Plebanski *et al.*, 1997b, Plebanski *et al.*, 1999). This therefore represents a form of tolerance induction / anergy, although the mechanism is quite distinct.

7.3

TRAP as a Vaccine Candidate

The use of circumsporozoite protein as a vaccine candidate is therefore fraught with problems, mainly in relation to it's extensive polymorphism in immunodominant T cell epitope regions. One way to overcome this problem is to use only conserved epitopes within CS protein, however few have been identified (Nardin *et al.*, 1989, Aidoo *et al.*, 1995, Chatterjee *et al.*, 1995). It is thus felt that alternative less polymorphic pre-erythrocytic vaccine candidates should be sought. TRAP is one such alternative, although no CD4 T cell epitopes had been identified for TRAP, and the nature of natural T cell immunity to TRAP was not known. I therefore carried out a detailed analysis of T cell responses to TRAP in naturally exposed Africans, in order to assess it's potential as a pre-erythrocytic vaccine candidate.

7.3.1

CD4 T Cell Epitopes

I first screened Gambian and Kenyan adults for the presence of $CD4^+$ T cell epitope regions within TRAP, and was surprised to find that they were distributed along the entire length of the molecule, with no obvious regions of immunodominance. Moreover, 65% of Kenyan adults responded to one or more epitope within TRAP, although reactivity in The Gambia was somewhat lower at 32%. The fact that TRAP stimulated responses to

peptides along it's entire length in multiple naturally exposed donors supports the use of the whole antigen in a vaccine construct.

7.3.2

Variant Cross-Recognition

TRAP is regarded as less polymorphic that CS protein, however numerous non conservative point mutations do arise along the length of the antigen (Robson *et al.*, 1990, Robson *et al.*, 1998). The effect that these have on T cell immune recognition was not known. I therefore assessed for cross-recognition for a number of naturally occurring variants of commonly recognised TRAP T cell epitopes in Kenyan adults. Disappointingly, there was no more cross-recognition than was observed to polymorphic variants of CS protein and thus single strain vaccination will not provide heterologous protection for polymorphic epitope regions. However, I identified 16 T cell epitopes within TRAP that are conserved for all sequences so far typed, and there was a tendency for conserved regions to be the most frequently recognised in the cross-sectional analysis of 217 Kenyans. These conserved epitopes might thus be incorporated into a multi-epitope vaccine which could provide cross-strain protection if these epitopes are indeed protective.

7.3.3

Correlates of Protection

Few studies of pre-erythrocytic T cell immunity to malaria antigens have found correlates of protection. There are 2 studies hinting at a protective effect for proliferative responses to the immunodominant Th2R CD4 T cell epitope region of CS protein (Riley *et al.*, 1990, Hoffman *et al.*, 1989b), and one study that found correlates of protection for cultured IFN-γ responses to LSA-1 (Luty *et al.*, 1999).

Since IFN-γ is the cytokine by which T cells mediate their protective, I assessed for the protective efficacy of IFN-γ TRAP responses assessed by *ex-vivo* ELISPOT assay in 217 Kenyan donors. This is the first study using the IFN-γ *ex-vivo* ELISPOT assay to assess for correlates of protection, but unfortunately no protection was found. Donors were

analysed for a protective association for time to first parasitaemia, and number of episodes of parasitaemia over 2 and 6 months. The reasons for a lack of protection include the possibilities that CD4 T cell responses to TRAP are not protective, that *ex-vivo* ELISPOT responses are not protective, or that I asked the wrong questions or failed to assess enough donors. All these issues are discussed in the previous chapter, and will need to be addressed in future studies.

Since I now know that *ex-vivo* ELISPOT, cultured ELISPOT and proliferation assays detect different memory T cell subsets, then the former 2 assays both need to be analysed carefully for correlates of protection. Proliferative responses to TRAP have also yet to be analysed for protective efficacy in malaria exposed donors. Perhaps the combined responses to different T cell assays will be more likely to correlate with protection, since it will give an overall picture of reactivity to the immunogens being assessed. Thus, future field studies for protection might consider using all 3 T cell assays.

7.3.4

East vs West Africans

One problem in the development of T cell inducing malaria vaccines is that different populations exhibit widely varying HLA types, particularly in Africa (Hill *et al.*, 1992a). All T cell responses are HLA restricted to a greater or lesser extent, and therefore any vaccine construct must contain sufficient epitopes to cover the broad range of HLA types in the population(s) to be vaccinated. I investigated what effect disparity in HLA types might have on the nature of T cell responses to TRAP, by performing similar studies in 2 African populations, The Gambia in West Africa and Kenya in East Africa. I found that the repertoire of T cell responses for the 2 populations was different. Repertoire differences between East and West Africans were also observed to both CS protein and the blood stage antigen MSP-1 (Lee *et al.*, 2000), suggesting that HLA differences may well have influenced the T cell repertoire to these malaria antigens.

The number of donors reacting to TRAP varied between the 2 countries being higher in the Kenyan study (62% reactivity) carried out in a region of higher malaria transmission compared to The Gambian response rate (32% reactivity). Interestingly, the reactivity rate to CS was comparable between the 2 populations, suggesting that CS protein might be susceptible to certain negative regulatory influences that do not affect TRAP responses. If it is true that TRAP responses are less easily suppressed *in vivo* this would be another good reason to use TRAP in malaria vaccines. The magnitude of response in terms of T cell precursor frequency levels were comparable between East and West Africa to all antigens tested, but were low as is typical for malaria T cell responses. It therefore seems that when responses were stimulated they were of a similar magnitude.

7.3.5

Adults vs Children

The repertoire differences observed above between 2 populations may not simply be explained by differing HLA types since subgroups within the same Kenyan population (adults and children) also gave a different repertoire of T cell responses, despite being of the same ethnic group and HLA distribution. The fact a child as young as 2 months of age had a T cell responses to TRAP in my study is encouraging since these potentially protective responses might be susceptible to boosting by vaccination. Indeed, by one year of age the rate of T cell reactivity to TRAP derived peptides was comparable to that observed in adults. Interestingly, the only group that had significantly lower precursor frequency levels was the under one year olds, and thereafter levels were comparable across all age groups. This suggested that a pre-erythrocytic vaccine might be suitable for very young children, which is encouraging since they are the main target population for malaria vaccination.

7.3.6

Th2 T Cell Responses

Few studies have comprehensively assessed Th2 T cell reactivity to pre-erythrocytic antigens. IL-4 and IL-10 ELISPOT assays were thus used to assess for Th2 type T cell

responses to TRAP derived peptides in Gambians and Kenyans. No IL-10 reactivity was observed in the Gambians, but more than 60% of Kenyan adults produced IL-10 to pools of peptides spanning the whole of TRAP. This needs to be borne in mind for vaccines incorporating the whole of the TRAP antigen, since IL-10 is an immunosuppressive cytokine and may dampen protective responses at the liver stage of infection (Le Moine *et al.*, 1999).

I could find no evidence that TRAP variant epitopes might use the same APL evasion strategy demonstrated for CS protein (ie differential induction of IFN-γ and IL-10 by variants of the same region). If TRAP is not susceptible to APL, this could be one reason why CS protein specific T cell reactivity rates were lower than TRAP reactivity in Kenya. This will need to be investigated further since APL antagonism can occur by means other than IL-10 induction, including the differential secretion of IL-4 (Evavold and Allen, 1991). Indeed, IL-4 reactivity levels of 33% were observed to TRAP peptide pools in Kenyans. A 17% reactivity to the same TRAP peptide pools was observed in Gambians by *ex-vivo* IL-4 ELISPOT, although the donor numbers were too small to draw any firm comparative conclusions. The high levels of IL-10 reactivity to TRAP observed in the Kenyan study may be a reflection of the fact that the blood samples were obtained during a period of peak malaria transmission. Indeed, high levels of IL-10 induction has been documented in malaria infected children and adults in Gabon (Winkler *et al.*, 1998, Winkler *et al.*, 1999).

7.4

Final Comments

Throughout this thesis I have studied T cell responses to two major pre-erythrocytic vaccine candidates, namely circumsporozoite (CS) protein and thrombospondin related adhesive protein (TRAP). I have identified a number of mechanisms that might explain the well recognised phenomenon of T cell nonresponsiveness in naturally exposed donors, which relate to CS protein in particular. I have carried out detailed studies of natural T cell immunity to TRAP, both in Kenya and The Gambia, and taken the opportunity to compare T cell responses to this antigen both between (Kenya vs Gambia) and within

(adults vs children) populations. I looked for correlates of protection between *ex-vivo* IFN-γ ELISPOT responses to TRAP and the subsequent development of malaria, but could find no protective effect. My findings illustrate the complexity of pre-erythrocytic stage T cell immunity in naturally exposed donors, and demonstrate that we still have a considerable way to go before we fully understand the nature of T cell immunity to this highly complex disease.

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